POLLEN BIOLOGY IN RELATION TO ARTIFICIAL HYBRIDIZATION IN THE GENUS *PROTEA*

by

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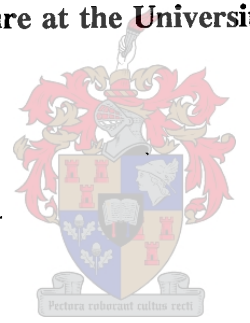

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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SUMMARY

Effects of pH, sucrose, boric acid and temperature on *in vitro* germination of pollen of *Protea repens* (L.) L. cv. 'Embers' were investigated in hanging-drop culture to establish optimum conditions for germination. Optimum values were found within ranges pH: 5 – 8, sucrose concentration: 0.4 – 0.7 M, boric acid concentration: 50 – 500 mg.ℓ^{-1}, and incubation temperature: 5 – 30°C. Storage temperature and humidity on pollen viability was studied in four *Protea* clones. Pollen was stored at a range of temperatures and relative humidities for up to one year and tested for ability to germinate *in vitro*. Pollen of *P. repens* cv. 'Sneyd', *P. eximia* cv. 'Fiery Duchess' and *P. magnifica* clone 'T 84 07 05', stored in liquid nitrogen (-196°C) and in a freezer (-14° to -18°C), retained a germination percentage as high as that of fresh pollen regardless of humidity. The study showed that long-term storage of protea pollen is not feasible at temperatures above 0°C. The correlations between the fluorochromatic reaction (FCR) and germinability were found to be low and nonsignificant. Fifteen month old cryopreserved 'Sneyd' pollen was shown to retain its ability to fertilize and set seed equal to that of fresh pollen. 'Sneyd', 'Fiery Duchess' and 'T 84 07 05' pollen could be repeatedly thawed and frozen in liquid nitrogen before its germinability *in vitro* decreased. The morphology and size of *Protea* pollen was studied, using light and scanning electron microscopy. Polymorphic grains were observed in two interspecific hybrids. Very small differences in pollen grain size were recorded between clones/species. The male fertility of 25 interspecific *Protea* hybrids, based on *in vitro* pollen germinability, was investigated. The majority of hybrids were found to be sufficiently fertile to be used in a breeding programme. Pistil structure and pollen tube pathways were investigated in 'Sneyd' using light and scanning electron microscopy. The pistil had four distinct regions, consisting of the stigma, the vertebra-shaped upper style, the heart-shaped lower style, and the ovary. The pistil had a stylar canal along its entire length and this canal was also the route by which pollen tubes grew to the ovary. Very low numbers of pollen tubes reached the ovary. The breeding system of 'Sneyd' and 'Fiery Duchess' were determined from pollen tube and seed set data, after controlled hand-pollinations. Both clones were found to be fully self-compatible. Very low percentages autogamous seed set were recorded. Interspecific crosses had a low success rate. An incompatibility reaction probably occurred on the stigma and/or

in the upper style regions.The attainment of maximum stigma receptivity of two *Protea* cultivars was investigated by means of seed set experiments, pollen tube growth observations and measurement of the degree of opening and closing of the stigmatic groove. Both cultivars were found to be protandrous. The maximum stigmatic groove width of both cultivars never exceeded the pollen grain diameter. It was concluded that *Protea* spp. must be hand-pollinated two to six days after anthesis in order to obtain maximum seed set; while for the observations of pollen tubes in the ovary, inflorescences must not be harvested before seven days after pollination.

OPSOMMING

Ten einde 'n optimale medium vir die *in vitro*-ontkieming van *Protea*-stuifmeel te ontwikkel, is die effek van pH, sukrose, boorsuur, en temperatuur op die *in vitro*-ontkieming van *Protea repens* (L.) L. cv. 'Embers'-stuifmeel deur middel van die hangdruppel-metode ondersoek. Die volgende reekse van veranderlikes wat getoets is, is as optimaal gevind; pH: 5 – 8, sukrosekonsentrasie: 0.4 – 0.7 M, boorsuurkonsentrasie: 50 – 500 mg.ℓ^{-1} en inkubasietemperatuur: 5 – 30°C. Die invloed van bergingstemperatuur en humiditeit op stuifmeel-lewenskragtigheid is in vier *Protea*-klone ondersoek. Stuifmeel is gestoor by 'n reeks temperature en relatiewe humiditeite vir tot een jaar, en vir *in vitro*-ontkiemingsvermoë getoets. Stuifmeel van *P. repens* cv. 'Sneyd', *P. eximia* cv. 'Fiery Duchess', en *P. magnifica* kloon'T 84 07 05', in vloeibare stikstof (-196°C) en in 'n vrieskas (-14° tot -18°C) geberg, het 'n ontkiemingspersentasie gelykstaande aan dié van vars stuifmeel gehandhaaf, ongeag van die humiditeit. Hierdie studie het verder aangetoon dat langtermynberging van *Protea*-stuifmeel bokant 0°C nie die moeite werd is nie. Die korrelasie tussen die fluorochromatiese reaksie (FCR) en ontkieming was laag en nie betekenisvol nie. 'Sneyd'-stuifmeel wat vir 15 maande in vloeibare stikstof gestoor is, het die bevrugtings- en saadsetvermoë gelykstaande aan vars stuifmeel behou. 'Sneyd', 'Fiery Duchess' en 'T 84 07 05'-stuifmeel kon herhaaldelik in vloeibare stikstof gevries en ontdooi word voordat hul ontkiemingsvermoë afgeneem het. Die morfologie en grootte van *Protea*-stuifmeel is deur middel van lig- en skandeerelektronmikroskopie bestudeer. Polimorfiese stuifmeelkorrels is in twee interspesie-hibriede waargeneem. Baie klein verskille in stuifmeelkorrelgroottes het tussen klone/spesies voorgekom. Die manlike vrugbaarheid van 25 *Protea*-interspesiehibriede, gebaseer op die *in vitro*-ontkiembaarheid, is ondersoek. Dit is gevind dat die meerderheid hibriede 'n voldoende graad van vrugbaarheid het om in 'n teelprogram te gebruik. Die stamperstruktuur en stuifmeelbuisweë in *P. repens* is deur middel van lig- en skandeer-elektronmikroskopie ondersoek. Die stamper bestaan uit vier kenmerkende gebiede, naamlik die stempel, die werwelvormige bo-styl, die hartvormige onderstyl, en die vrugbeginsel. Die stamper het 'n stylkanaal regdeur die totale lengte van die stamper, en hierdie kanaal is ook die weg waarvolgens stuifmeelbuise na die vrugbeginsel

gegroei het. Min stuifmeelbuise het die vrugbeginsel bereik. Die teelsisteem van 'Sneyd' en 'Fiery Duchess' is deur middel van stuifmeelbuis- en saadsetdata ná gekontroleerde handbestuiwings ondersoek. Beide klone was ten volle selfverenigbaar. Die persentasie outogame saadset was baie laag. Interspesiekruisings het 'n baie lae sukses gehad. Dit is voorgestel dat die onverenigbaarheidsreaksie in die stempel en/of in die bopunt van die styl plaasvind. Die bereiking van maksimum stempelontvanklikheid van twee *Protea*-cultivars is deur middel van saadseteksperimente, stuifmeelbuisdata en waarnemings van die oop- en toemaak van die stempelgroef ondersoek. Beide cultivars was protandries. Die maksimum stempelgroefwydte het nooit die stuifmeelkorreldeursnee oorskry nie. Dit is afgelei dat *Protea*-spesies twee tot ses dae na antese handbestuif moet word vir optimale saadset. Vir die waarneming van stuifmeelbuise in die vrugbeginsel, moet bloeiwyses nie voor sewe dae na bestuiwing geoes word nie.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 General introduction

The entire Proteaceae family comprises approximately 1 500 ancient woody evergreen species, that was represented in Gondwanaland before the separation of the Australian and African continents 130 million years ago (Johnson and Briggs, 1975; Vogts, 1982). The genus *Protea* consists of about 114 species; of these, 82 occur in Africa south of the Limpopo, and 35 in tropical Africa, north of the Limpopo, while three species are common to both regions (Rourke, 1980). *Protea* form the most important commercial component of the South African wild-flower (fynbos) industry, but only about 15 species are of great commercial importance. *Protea* spp. to the value of approximately R20 million are exported annually from South Africa. In addition to those areas in which they originated, *Protea* spp. are cultivated in a number of countries, including Israel, USA, New Zealand, Australia and Zimbabwe.

The most characteristic feature of this genus is the structure of the inflorescence, consisting of a dense mass of hermaphrodite florets (45 – 1024) arranged around a central woody core (Collins and Rebelo, 1987). The florets open over a period of seven to 14 days (Brits and Van den Berg, 1990). It is these spectacular and unique inflorescences which give some of the species the potential for use as cut flowers and in landscaping.

To meet the requirements of fashion-conscious markets, systematic controlled breeding programmes are essential in order to produce novel products. However, severe bottlenecks in current breeding technology have been encountered, which include crossing incompatibility between species, lack of pollen storage techniques, and low hybrid seed set (Brits, 1992).

This study was undertaken to try and overcome some of these bottlenecks experienced with *Protea* breeding. Instrumental to this is a basic-level study of *Protea* reproductive biology. For practical reasons, the study was restricted to four important *Protea* species, chosen according to the following criteria: economic value, availability and taxonomic distance.

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CHAPTER 2

LITERATURE REVIEW

2.1 Methods of testing pollen viability

The need to test the functional quality of pollen arises in many contexts, for example, in assessing the fertility of parent plants and hybrids in plant breeding and genetical experimentation, and in monitoring pollen state during storage. Pollen viability is generally defined as the ability of the pollen grain to perform its function of delivering sperm cells to the embryo sac following compatible pollination (Shivanna *et al.*, 1991). Assessment of pollen viability in this sence is cumbersome, time consuming, and not always feasible (Heslop-Harrison *et al.*, 1984). Hence, a considerable number of short-cut methods of measuring pollen viability have been introduced. Most of these methods provide a good indication of the germination potential of the pollen but do not measure the capacity of the male gametophyte to develop a pollen tube. Standard tests of viability involve pollen germination *in vivo, in vitro* and the direct assay of viability of nongerminated grains through histochemical methods. The merits of each of these methods will be briefly discussed in the light of the latest experimental results.

2.1.1 *The* in vivo *approach*

Most researchers agree that the ultimate functional test involves placing the pollen on the stigmas of emasculated flowers and observing the number of pollen tubes in squashed styles (Dempsey, 1962; Papademetriou, 1974; Widrlechner *et al.*, 1983) or the number of seeds in the mature fruit (McGuire, 1952; El Ahmadi and Sevens, 1979; Dickson and Boettger, 1984; Grigg *et al.*, 1988). These methods are time-consuming and therefore impractical for testing many samples. Furthermore, seed set may depend not only on fertilization, but also on the post-pollination development of the ovary, stigma receptivity, and incompatibility reactions (Heslop-Harrison *et al.*, 1984). Staining techniques (Nair and Narasimhan, 1963) and a more sensitive fluorescence technique (Martin, 1959) have been developed to view pollen tubes that penetrated the style. Stanley and Linskens (1974) summarized the main difficulties arising in the use of *in vivo* techniques to assay pollen viability. They concluded that the method is preferable only when all potential sources of interference can be recognized.

2.1.2 *The* in vitro *approach*

In vitro germination involves germinating the pollen on artificial media and observing, under a microscope, the percentage of grains producing tubes after a given time. This figure is considered an index of viability of the pollen sample (Polito and Luza, 1988a; Parfitt and Ganeshan, 1989; Shivanna *et al.*, 1991; Abdul-Baki, 1992). The procedure requires much less time than the *in vivo* approach and is adaptable to routine screening of many samples. Stanley and Linskens (1974) summarized five simple methods regularly used, namely the hanging-drop, spot test, well, agar and membrane assays. The hanging-drop method, first introduced by Van Tieghem (1869), is the most commonly used method for germinating micro-amounts of pollen to determine viability and involves a drop of media containing pollen inserted in a circular chamber or closed area suspended over water.

The *in vitro* approach assumes that optimum conditions have been established so that germination approximates that on the plant. However, most pollen tubes cultured *in vitro* stop growing before reaching the length normally attained in the style. This suggests that optimum growth conditions are not always established adequately in *in vitro* media (Visser, 1955). Difficulties in establishing optimum conditions for pollen grain germination are due to interactions between the effects of components as will be discussed in section 2.2 of this review.

Erratic pollen germination in artificial environments makes the germination test unreliable (King, 1960). However, many comparative studies (e.g., Werner and Chang, 1981; Pearson and Harney, 1984; Parfitt and Ganeshan, 1989; Shivanna *et al.*, 1991) have led to reliable prescriptions for germination. Germinability tests have the potential for providing the best basis for predicting pollen performance (Heslop-Harrison *et al.*, 1984).

2.1.3 *The histochemical approach*

The histochemical approach is based on the ability of the vegetative cell of the pollen grain to stain specific constituents of that cell, or on the activity of specific enzymes. This approach to determining pollen viability requires less time, thus making it suitable for routine

screening of many samples. Heslop-Harrison *et al.* (1984) identified the following three methods in the histochemical approach:

2.1.3.1 Stainability tests

These tests stain pollen with dyes which are used as indices of viability. The chemicals are adsorbed to specific cell constituents only present in mature pollen. The following stains have been frequently used: **acetocarmine** and **safranin**, which stains chromatin and ribonucleic acids (Sarvella, 1961; Pearson and Harney, 1984; Parfitt and Ganeshan, 1989); **aniline blue in lactophenol**, which stains starch and other polysaccharides (Sarvella, 1961; Hauser and Morrison, 1964; Werner and Chang, 1981; Grigg *et al.*, 1988); **phyloxin-methyl green**, which stains both cytoplasm and cellulose (Owczarak, 1952; Weaver and Timm, 1989); **iodine potassium iodide** (IKI), which stains starch (Werner and Chang, 1981); and **Alexander's stain**, which stains cell walls, protoplasm, and cytoplasm (Alexander, 1969; De Wet and Robbertse, 1986; Parfitt and Ganeshan, 1989). Other stains used have been acetic orcein, acid fuchsin, propiono-carmine etc.

The suitability of these stains as indicators of pollen viability has been examined in various species in recent years and their validity has been seriously questioned. Acetocarmine provided only a rough estimate of rose pollen viability (Pearson and Harney, 1984). One disadvantage of acetocarmine and aniline blue in lactophenol is that they do not stain the aborted pollen at all, and do not produce satisfactory staining of thick-walled pollen like that of *Gossypium, Hibiscus, Silphium* and *Cucurbita* (Alexander, 1969). Heslop-Harrison *et al.* (1984) evaluated several commonly used stains (e.g., acetic orcein and acid fuchsin) and found a low correlation between stainability and pollen germinability in *Lilium, Narcissus, Helleborus, Primula, Medicago* and *Lycopersicum*. Acetocarmine and Alexander's stain was found to stain dead *Prunus* pollen (Parfitt and Ganeshan, 1989). These workers concluded that pollen staining procedures are not reliable or consistent and are not positively correlated with *in vitro* assays. Contrary to the findings of Hauser and Morrison (1964), Werner and Chang (1981) found that correlations between pollen staining percentage and germination percentage were low and nonsignificant for aniline blue, IKI and propiono-carmine in *Prunus persica*.

The search for new and better pollen viability stains still continues, and, recently, Gowda *et al.* (1991) have described a stain extracted from the berries of *Cocculus hirsutum* that gave positive results in pollen fertility studies in many crops.

In general, most specific stains are supposed to distinguish between pollen grains with and without cytoplasm, but the major problem associated with their use is that "Pollen without cytoplasm are certainly sterile, but those with cytoplasm are not necessarily fertile" (Ockendon and Gates, 1976).

2.1.3.2 Enzyme tests

These tests are based on the activity of various functional enzymes of the vegetative cell. They do not depend merely on the presence or absence of substrate or chemical constituents (Stanley and Linskens, 1974). Most of the tests are based on the use of so-called **redox dyes**, first used to test viability of pollen by Sharadakov in 1940, using **benzidine** to measure peroxidase activity (Stanley and Linskens, 1974). Benzidine was reintroduced by King (1960) who found consistent viability comparisons even among dated pollens of *Solanum, Lycopersicon, Ipomoea* and *Saccharum*. However, because of the non-specificity and variability of staining reactions for pollen of different taxa (Hauser and Morrison, 1964), it has not been widely used.

The most commonly used reagents for testing enzyme activity are those of the tetrazolium group (Oberle and Watson, 1952; Aslam *et al.*, 1964; Visser *et al.*, 1977; Widrlechner *et al.*, 1983; Heslop-Harrison *et al.*, 1984; Parfitt and Ganeshan, 1989; Abdul-Baki, 1992). **2, 3, 5 — Triphenyl-tetrazolium chloride** (TTC) has been most widely used and its value was investigated in detail by Smith (1951) and Cook and Stanley (1960). TTC was found to be of no value as an indicator of pollen viability in peaches, pears, apples and grapes (Oberle and Watson, 1952). However, Widrlechner *et al.* (1983) working with deciduous azalea pollen, Werner and Chang (1981) with *Prunus persica* pollen, Sarvella (1961) and Aslam *et al.* (1964) with cotton pollen, have all reported that TTC provided a quick and reliable index of pollen viability.

Other indicator dyes in the tetrazolium group have also been reported as useful in pollen viability tests. Hauser and Morrison (1964) found **nitroblue tetrazolium** (NBT) to be a good index of viability in 13 taxa of angiosperm pollen. However, Werner and Chang (1981) reported low and nonsignificant correlations between germination and pollen staining percentages with NBT for *Prunus persica* pollen. **2,5-diphenyl tetrazolium bromide** (MTT) (Widrlechner *et al.*, 1983) and **Tetrazolium red** (Sarvella, 1961) have also been found to have promise as indicators of pollen viability. **3-Amino -9 ethylcarbazole** (AEC) gave a very accurate prediction of azalea pollen viability (Widrlechner *et al.*, 1983).

2.1.3.3 The FCR test

Another histochemical approach, suggested some years ago by Heslop-Harrison and Heslop-Harrison (1970) as a test for pollen viability is the **fluorochromatic reaction** (FCR) which tests for the presence of an active esterase and the integrity of the plasmalemma of the vegetative cell (Heslop-Harrison *et al.*, 1984). Application of the test uses the substrate **fluorescein diacetate** (FDA). Technically, the test is very simple to apply.

Heslop-Harrison and Heslop-Harrison (1970) observed fluorochromasia in the fresh pollen of some 30 flowering plant species and concluded that it may have unique advantages in the testing of pollen viability. FDA subsequently proved an excellent test for pollen viability in a number of different species (Ockendon and Gates, 1976; Shivanna and Heslop-Harrison, 1981; Heslop-Harrison *et al.*, 1984; Shivanna *et al.*, 1991; Peterson and Taber, 1987; Grigg *et al.*, 1988; Jansson and Warrington, 1988; Radicati Di Brozolo *et al.*, 1990; Pinney and Polito, 1990). Griessl (1989), using the contrast-fluorochromatic staining technique (fluorescein-diacetate/propidium-iodine), obtained excellent results in the 18 taxa he examined. The combination of fluorochromes enabled him to easily differentiate fluorescing live pollen grains from dead pollen grains, the latter staining red. Abdul-Baki (1992) developed a fast and reliable method for testing pollen viability through the combined use of FDA and *in vitro* approaches in *Lycopersicon esculentum*. In contrast, Widrlechner *et al.* (1983) found that FDA staining provided the least accurate estimate of pollen germination in deciduous Azaleas.

FDA is effective when used with fresh pollen, but Heslop-Harrison *et al.* (1984) experienced problems when using the test on stored pollen. The FCR test is subject to many limitations which are discussed in detail by Heslop-Harrison *et al.* (1984). Notwithstanding these limitations, Heslop-Harrison and Heslop-Harrison (1970) concluded that "the evaluation of pollen quality by the FCR may not therefore provide a test of pollen fertility in the full sense, but it probably comes nearer to doing so than most other tests currently available".

2.1.4 *Conclusions*

1. Almost all histochemical methods tend to overestimate pollen viability.

2. Before a reliable assessment of pollen quality can be made, the percentage germination, viability and stainability should be subjected to an analysis of correlation.

3. Different methods should be judged on their relative merit and purpose of evaluation. The purpose for which pollen is tested, and the required accuracy, versus technical complexity and cost should be ascertained.

4. Considering the literature available on the subject, no absolute conclusion can be made as to which approach or method of pollen viability testing will be the most accurate assessment of pollen quality. However, the ranking on a theoretical basis would probably be in the order:

in vivo approach > *in vitro* approach > FCR tests > enzyme tests > stainability tests.

5. Furthermore, it appears that none of the histochemical methods takes **pollen vigour** into account.

2.2 Factors effecting the *in vitro* germination of pollen: Formulation of an optimum medium

Many physical and chemical factors affect pollen germination *in vitro*, and this may explain the often erratic responses of pollen to artificial environments. It is not only the medium composition that is critical for pollen germination but other factors, such as flower developmental stage, temperature and humidity ranges before anthesis, type of pollen

(binucleated *vs*. trinucleated) and hydration etc. that have a pronounced effect on pollen germination *in vitro*. However, this discussion will be confined to factors directly related to the medium as such.

2.2.1 *Effect of carbohydrates*

The role of carbohydrates in the germination medium has been postulated as, firstly, its osmotic properties of regulation of water uptake and thereby controlling bursting of pollen (Visser, 1955; Brewbaker and Majumder, 1961; Vasil, 1964; De Bruyn, 1966a; Kim *et al.*, 1985); and , secondly, its nutrient source properties for pollen tube growth (Visser, 1955; De Bruyn, 1966; Sahar and Spiegel-Roy, 1984). However, Vasil (1964) and Leduc *et al.* (1990) propose that sugar functions mainly as an osmoticum rather than as energy source.

Sucrose has been the most generally employed carbohydrate for the *in vitro* germination of pollen (Pearson and Harney, 1984; Parfitt and Ganeshan, 1989; Abdul-Baki, 1992). The concentration of sugar which has been employed in germination media varies widely. Van Tieghem (1869) and others have reported that pollen of approximately 30 genera germinate readily in water (0% sugar). In contrast, Branscheidt (1930) obtained germination of *Cornus, Helleborus* and *Cannabis* pollen only in sugar concentrations of 70 – 80%. Generally, sugar concentrations above 40% impair pollen tube growth, while those below 2% lead to increased bursting of the tubes (Brewbaker and Majumder, 1961). For most plant species the optimum sucrose concentration can be defined only within a rather broad range (Visser, 1955; Kim *et al.*, 1985; Leduc *et al.*, 1990; Pinney and Polito, 1990).

Other carbohydrates have also been used with varying results. In comparative studies on a number of mono- and oligosaccharides, sugar alcohols and sucrose, indicate best germination with sucrose (De Bruyn, 1966a; Kim *et al.*, 1985; Klein, 1990). However, De Lange and Boucher (1993) found that sucrose, fructose, galactose, raffinose, mannitol and sorbitol, when used as single carbohydrate sources, resulted in hardly any *in vitro* germination of *Audounia capitata* pollen. A composite medium consisting of sucrose, fructose and glycerol proved to be the best carbohydrate source in this case.

2.2.2 *Effect of boron*

The exact role of boron in pollen germination has not been elucidated. However, there is no doubt that the promotive influence of boric acid is not due to its acidity since borax is just as effective (Schmucker, 1935; Vasil, 1960). According to Dugger (1983), boron may be involved in pollen tube wall biosynthesis, increasing respiration and facilitating sugar absorption and metabolism.

Since Schmucker (1933) demonstrated the role of boron in pollen germination, many investigators (Thompson and Batjer, 1950; Visser, 1955; Brewbaker and Kwack, 1963; Pfahler, 1965; De Bruyn, 1966b; Visser *et al.*, 1977; Dickenson, 1978; Sahar and Spiegel-Roy, 1984; Luza and Polito, 1985), have reported that small amounts of boric acid or various other boron compounds (Stanley and Lichtenberg, 1963; De Bruyn, 1966b) are either essential for or beneficial to, pollen germination and tube growth of many species. For those species tested, the optimum concentration varied between 10 and 40 mg.ℓ^{-1} (Visser, 1955).

In other plant species boric acid appears not to be essential for pollen germination (Schmucker, 1935; Vasil, 1964; Kim *et al.*, 1985; Leduc *et al.*, 1990). Zielinski (1968) reported that boron at levels of 25, 50 and 100 mg.ℓ^{-1} completely inhibited filbert pollen germination in culture solutions. Dickenson (1978), working with *Lilium longiflorum* pollen and Visser (1955) with pear and apple pollen, have both found that borate affects only the rate of pollen tube growth and not the time of tube initiation. The experiments carried out by Visser (1955) with pear pollen, showed that the degree of boron sensitivity of the pollen *in vitro* is related to the boron level of the plant and that maximal germination and tube growth can be obtained with boron concentrations many times higher than the minimum (Visser, 1955; De Bruyn, 1966b; Dickinson, 1978). Visser (1955) observed that the degree of boron sensitivity of pear and apple pollen increases with increasing pollen age when stored. Boric acid interacts complexly with other constituents of the germination medium such as temperature and sugar concentration.

2.2.3 *Effect of calcium*

The physiological role of calcium is discussed in detail by Brewbaker and Kwack (1963, 1964). Primarily, calcium participates in the formation of the pollen cell wall. Tip growth of pollen tubes depends upon having optimal amounts of calcium present, and is inhibited by the presence of either excessive or insufficient levels of Ca^{2+} in the culture medium (Picton and Steer, 1983). During the germination process, calcium is released into the medium (Brewbaker and Kwack, 1963).

In studying the "population effect" (see section 2.2.4) in pollen germination, Brewbaker and Kwack (1963, 1964) concluded that calcium is an essential requirement for tube growth of pollen in many, if not all, plant species. This requirement has been reported for many species, including *Setaria sphacelata* (De Bruyn, 1966b); *Lilium longiflorum* (Dickinson, 1978); *Persea americana* (Sahar and Spiegel-Roy, 1984); *Simmondsia chinensis* (Lee *et al.*, 1985); *Juglans regia* (Luza and Polito, 1985) and *Capsella bursa-pastorus* (Leduc *et al.*, 1990). However, the addition of calcium to the medium did not change the percentage germination of rye pollen and significantly decreased the length of the pollen tubes produced (Pfahler, 1965).

The calcium source which has been used most often is calcium nitrate (Heslop-Harrison *et al.*, 1984; Shivanna *et al.* 1991). De Bruyn (1966b) found that at low and optimal levels, calcium and boron appeared to act synergistically upon tube growth of *Setaria sphacelata* pollen. Moreover, De Bruyn (1966b) found that calcium had practically no effect in the absence of boron. Brewbaker and Kwack (1963) found that magnesium improved the effects of calcium on tube growth. Therefore, calcium, while an essential requirement for pollen germination on its own, also requires the presence of other elements for optimum effect.

2.2.4 *Effect of pollen density*

The density of pollen in the germination medium, when using the hanging drop technique, has a striking effect on both the germination and tube growth of pollen. A minimum concentration of pollen grains must be placed in a given volume of solution if maximum

germination is to be obtained (Visser, 1955; Brewbaker and Kwack, 1963). This described phenomenon, termed the "population effect", "mass effect" or "mutual stimulation effect" has been studied in detail by a number of investigators and was found to be universal in its occurrence (Visser, 1955; Brewbaker and Majumber, 1961; Brewbaker and Kwack, 1963, 1964; De Lange, pers. comm.; Leduc *et al.*, 1990; Egea *et al.*, 1992). Visser (1955), working on apple and pear pollen, observed that the relationship between the germination percentage and the number of grains per unit of volume is approximated by a saturation curve which is linear for the greater part.

The mutual stimulation effect is not as strong in pollen spread evenly over agar or in solution media (Kwack, 1965; Stanley and Linskens, 1974), and the effect can be completely overcome by the addition of calcium to the medium, as was confirmed in 86 species representing 39 plant families (Brewbaker and Majumder, 1961; Brewbaker and Kwack, 1963; Kwack, 1965). The population effect was therefore explained as being a consequence of induced calcium deficiency (Brewbaker and Kwack, 1964).

2.2.5 *Effect of pH*

The optimum pH and viable pH ranges for pollen tube growth differs from species to species and reported ranges are wide (Kim *et al.*, 1985), lying between pH 5.0 and pH 7.0 (Brewbaker and Kwack, 1964; Dickinson, 1978; Tupý and Říhová, 1984; Kim *et al.*, 1985; De Lange, 1989; Leduc *et al.*, 1990; De Lange and Boucher, 1993). Filbert pollen (Kim *et al.*, 1985) and tobacco pollen (Tupý en Říhová, 1984) can also germinate under pH conditions < 5.0 but these conditions are sub-optimal. Leduc *et al.* (1990) found that extreme pH conditions of 3.0 and 9.0 are inhibitory to pollen germination in *Capsella bursa-pastoris*.

Kim *et al.* (1985) reported that *Corylus avellana* pollen germination showed a sharp decrease in the width of the viable pH range when the germination medium was supplemented with boric acid and/or calcium, magnesium and potassium, suggesting that there is a complex control of pH optimum width by external factors. The mode of action of these factors is not yet known.

Tupý and Říhová (1984) found that growing pollen tubes of *Nicotiana tabacum* acidify the surrounding medium and that this acidification results in arrested growth. However, De Lange (pers. comm.) has concluded that medium acidification in the case of *Citrus* pollen might not be important because of the relatively wide viable pH range in this species.

2.2.6 *Effect of temperature*

Optimum temperatures and viable ranges for *in vitro* pollen germination and pollen tube growth also varies widely among species. For most pollen species, this relationship can be represented by an optimum curve (Visser, 1955). The optimum temperature for germination and pollen tube growth in most investigations is between 20° and 30°C, e.g. *Brassica campestris*: 20°C (Kuo *et al.*, 1981); *Persea americana*: 25° – 27°C (Sahar and Spiegel-Roy, 1984); *Prunus persica*: 23°C (Weinbaum *et al.*, 1984); *Simmondsia chinensis*: 25° – 30°C (Lee *et al.*, 1985); *Actinidia deliciosa*: 22° – 28°C (Jansson and Warrington, 1988) *Carica papaya*: 22° – 26°C (Cohen *et al.*, 1989); *Rosa hybrida*: 23°C (Gudin *et al.*, 1991). However, Kim *et al.* (1985), working with *Corylus avellana*, Vasilakakis and Porlingis (1985) with *Pyrus communis* and Egea *et al.* (1992) with *Prunus armeniaca* have found that the optimum temperature for *in vitro* pollen germination was 15°C. Studies by Kuo *et al.* (1981), Jansson and Warrington (1988) and Cohen *et al.* (1989) have indicated that reduced pollen grain germination and pollen tube growth at very low or high temperatures are associated with an increase in the number of burst pollen grains.

Weinbaum *et al.* (1984), working with *Prunus dulcis* and *Prunus persica*, and Vasilikakis and Porlingis (1985) and Luza *et al.* (1987) with *Pyrus communis*, found that tube growth has a higher temperature requirement than the requirement for germination. Optimum temperatures for germination and tube growth in pear and apple, tend to be higher in the presence of boron (Visser, 1955).

Temperature during flower development strongly influences the germination of pollen at anthesis (Weinbaum *et al.*, 1984). These authors concluded that there is a positive linear correlation between staminate bloom date and optimum temperature for pollen germination. Higher optimum temperatures for germination were found to be associated with later

blooming cultivars. Moreover, Luza *et al.* (1987) found that no germination of pollen from any of the *Juglans regia* or *J. nigra* cultivars occurred below 14°C. However, Egea *et al.* (1992) have reported that in some *Prunus armeniaca* cultivars pollen grew as fast at 5°C as at other temperatures, demonstrating a possible adaptation to low temperatures during blossoming.

2.2.7 *Effect of macronutrients*

Other macronutrients (excluding calcium which has already been discussed in section 2.2.3) have also been found to have an effect on *in vitro* germination of pollen. Leduc *et al.* (1990) evaluated several macronutrients and found with *Capsella bursa-pastoris* pollen that the omission of potassium chloride, ammonium nitrate and potassium nitrate had no significant effect on germination. However, removal of magnesium from the medium significantly decreased germination of *Capsella* pollen. Similar results have been obtained with *Petunia inflata* and *Ornithogalum virens* (Brewbaker and Kwack, 1963). Moreover, Brewbaker and Kwack (1963, 1964) have found that the basic salts of K, Na or Mg, singly or together, can act to enhance calcium activity, thus acting as supporting ions for the calcium effect (overcoming the "population effect"). De Bruyn (1966b) found that low levels of magnesium stimulated germination, and particularly tube growth of *Setaria sphacelata*, but that higher levels were inhibitory. In contrast to magnesium, potassium and sodium did not greatly affect germination or tube growth of *S. sphacelata* pollen.

De Lange (pers. comm.) has found that the medium containing sucrose and agar only proved to be superior to that containing the macronutrients calcium, magnesium and potassium for *Citrus grandis* pollen. Sahar and Spiegel-Roy (1984) found that increased germination of *Persea americana* pollen was obtained when potassium was added to the solution. However, Lee *et al.* (1985) found that potassium had no affect on the percentage of *Simmondsia chinensis* pollen that germinated.

2.2.8 *Effect of micronutrients*

Micronutrients, other than boron (section 2.2.2) also play a significant role in the *in vitro* germination of pollen.

Leduc *et al.* (1990) found that micro-elements Mn, Zn, K, Na, Cu and Co were not essential to pollen germination of *Capsella bursa-pastoris*. Low concentrations of these elements were found to be slightly beneficial but increasing the concentrations resulted in a sharp decrease in germination. Similar results were obtained with *Areca catechu* pollen (Raghaven and Baruah, 1959).

De Bruyn (1966a) reported that although Cu, Mn, Zn and Mo could not replace boric acid, 1.0 mg.ℓ^{-1} of each of these elements (except Mo) stimulated germination and growth of *Setaria sphacelata* pollen in the presence of boron. Brewbaker and Kwack (1964) found that methyl donors, like Na, enhanced the calcium effect. Adding Mn to the germinating medium has been found to greatly improve the germination of plum pollen, by diminishing the amount of pollen bursting (Zielinsky and Olez, 1963). In contrast to this, Zielinsky (1968) found no significant effects of the addition of Mn on germination and tube growth of *Corylus* pollen.

2.2.9 *Effect of hormones and vitamins*

A few studies have investigated the role of hormones and vitamins on the *in vitro* germination of pollen. Hormones and vitamins improved the germination and tube length of nine species of pollen in the Cucurbitaceae (Vasil 1960). Usually extremely low concentrations are required. Raghaven and Baruah (1959) found similar results with the pollen of *Areca catechu*. De Bruyn (1966a) working with *Setaria sphacelata*, found that neither indolyl-acetic acid (IAA) nor gibberellic acid (GA) could replace boron, since no germination or tube growth occurred in its absence. Moreover, these compounds had no effect on germination or tube growth in the presence of boron, and both compounds were completely inhibitory at concentrations of 100 mg.ℓ^{-1}. *Lilium longiflorum* pollen germination and tube growth were not stimulated by auxin or gibberellin (Dickinson, 1978).

De Bruyn (1966a) also investigated the effects of eight vitamins on the *in vitro* germination of *Setaria sphacelata* pollen. Positive effects were observed with riboflavin and

calcium pantothenate, but thiamine, pyridoxine, nicotinic acid and ascorbic acid had very little effect on germination and tube growth. Vitamins B_1 and B_6 produced an increase in germination of *Capsella bursa-pastorus* pollen (Leduc *et al.*, 1990).

2.2.10 *Conclusions*

1. Optimum conditions for *in vitro* germination and tube growth of pollen vary widely among species and even among cultivars of the same species.

2. The effect of a particular compound also varies among different species and cultivars.

3. There is considerable interaction between the components of the germination medium.

4. *In vitro* pollen tube lengths are generally shorter than those attained *in vivo*. This can probably be accounted for by the fact that important unknown conditions for growth are absent from culture media.

5. Although many substances have an influence on the *in vitro* germination and tube growth of pollen, pollen of most species can be adequately germinated *in vitro* with a medium containing only sucrose, boric acid and a calcium source.

6. Except for sucrose, all the components of the *in vitro* germinating medium must be supplied at very low concentrations to avoid toxicity.

7. The aim of the germinating medium is to get maximum germination and not necessarily maximum pollen tube growth, when testing for pollen viability.

2.3 Pollen storage

Plant breeders often require immediate availability of germplasm for hybridization. Genotypic variability can be stored compactly as pollen. Establishment of pollen "banks" need definite protocols for maintaining pollen viability and fertility over extended periods.

A summary of pollen storage research prior to the 1950's was published by Visser (1955). The longevity of pollen depends on the extent to which storage conditions can reduce physiological functions without damaging the organism (Visser, 1955; Stanley and Linskens, 1974). Critical external factors include relative humidity, temperature and the atmosphere surrounding the pollen (Stanley and Linskens, 1974). These critical factors as well as specific storage techniques will be briefly discussed.

2.3.1 *Factors affecting viability in storage*

2.3.1.1 Humidity

The degree of humidity is the most important single factor affecting viability during storage (Stanley and Linskens, 1974). The large majority of pollens maintain viability best at low humidities (6 to 60%) (Allan, 1963; Vithanage and Alexander, 1985; Polito and Luza, 1988b; Pinney and Polito, 1990; Connor and Towill, 1993). Each pollen species appears to have an optimum relative humidity. However, optimum moisture levels necessary to maintain viability at different temperatures for most pollen species have not been determined (Connor and Towill, 1993). Extremes of humidity have detrimental effects on pollen viability and reduce the storage period (Allan, 1963). However, low moisture levels must be avoided in the storage of trinucleate pollen, e.g., graminaceous pollen, which must be stored at high relative humidities (80 – 100%) (Visser, 1955; Zielinski, 1968; Henny, 1980; Barnabás and Rajki, 1981). Frequent fluctuations of relative humidity during storage also cause a quick loss of viability (Stanley and Linskens, 1974). However, favourable results have been obtained without paying attention to the humidity during sub-zero storage (Visser, 1955; Cohen *et al.* 1989; Pinney and Polito, 1990; Osborne *et al.*, 1992) and humidity control apparently becomes less important as the temperature decreases (Visser, 1955; Hanna, 1990).

Humidity surrounding the pollen in storage affects viability by controlling the moisture content of the pollen. Sulphuric acid solutions of varying density (Stevens, 1916; Wilson, 1921; Solomon, 1951; Weast, 1988) and saturated solutions of various salts (Winston and Bates, 1960; Young, 1967; Henderson and Pixton, 1981) can be used for this purpose.

2.3.1.2 Temperature

In general, pollen longevity appears more influenced by variations in humidity than by variations in temperature but there are exceptions (Stone *et al.*, 1943). For most pollen species, longevity increases with decreasing temperature and is practically indefinite at a temperature of -196°C (Stanley and Linskens, 1974). Numerous studies on various species including *Corylus* (Zielinski, 1968); *Betula* (Alam and Grant, 1971); *Juglans* (Luza and Polito, 1985); *Simmondsia* (Lee *et al.*, 1985); *Vitis* (Ganeshan, 1985); *Pistacia* (Polito and Luza, 1988b); and *Carya* (Yates *et al.*, 1991), have shown that pollen storage at temperatures above 0°C, even with controlled humidity, is not feasible if the aim is to store pollen from one season to the next. Viability at room temperature declines very rapidly, especially when the humidity of the storage atmosphere is not controlled (Luza and Polito, 1985). The situation at temperatures below 0°C is somewhat different and viability can be extended for much longer periods. The storage of pollen at temperatures below 0°C will be discussed in more detail in section 2.3.2.1.

2.3.1.3 Atmosphere surrounding the pollen

Reduced air pressure has proved in some cases to have favourable and in other cases unfavourable results, and Visser (1955) concluded that it is doubtful if a low air pressure is of any value in pollen storage. Reduced pressure was harmful to *Pistacia* pollen viability (Stone *et al.*, 1943). Visser (1955) found with apple and pear pollen that at temperatures above 0°C, pollen retained its viability better at reduced than at normal air pressure, but no significant differences were obtained at temperatures below 0°C.

Reduction of the partial pressure of oxygen (lowering of the O_2 level) has proved in some cases to prolong the viability of pollen (Visser, 1955; Stanley and Linskens, 1974). Allan (1963), working with *Carica papaya* pollen, found that removal of CO_2 from the atmosphere around pollen had a depressing effect on viability. The longevity of cycad pollen in storage has been found to be better in an ambient atmosphere than in a nitrogen atmosphere (Osborne *et al.*, 1992). Sahar and Spiegel-Roy (1980) found that *Citrus* pollen viability can be maintained for long periods by a combination of an oxygen-free atmosphere and deep-freeze temperatures. They also found no significant differences between N_2 and CO_2 atmospheres.

2.3.2 *Methods of storing pollen*

2.3.2.1 Storage at temperatures below 0°C

Organic tissues, including pollen can be preserved at temperatures below 0°C (Barnabás and Rajki, 1981). Pre-drying before storage at temperatures below 0°C is essential for most pollen species to avoid freezing damage (Stanley and Linskens, 1974; Haunold and Stanwood, 1985; Hanna, 1990; Connor and Towill, 1993). For successful storage at low temperatures, moisture must be reduced so that at least the freezable water is removed (Connor and Towill, 1993).

Techniques for low-temperature storage have ranged from using household freezers (-20° C), low temperature deepfreezers (-80°C), nitrogen vapour phase (-140°C), freeze drying, and liquefied gases (-180° to -271°C). Generally, the lower the storage temperature, the longer pollen viability can be maintained. Some biochemical activity is apparently still possible at -20°C (Visser, 1955).

Although several earlier workers, including Visser (1955), found humidity control to be unnecessary at temperatures below 0°C, other workers (Olmo, 1942; Allan, 1963; Luza and Polito, 1985; Polito and Luza, 1988b; Hanna, 1990) found that controlling humidity during low-temperature storage had a marked effect on the retention of germinability of pollen. Hanna (1990) reported that in pearl millet, pollen moisture content was more critical for maintaining viability at -18°C than at -73°C.

Pollen from many species including *Vitis vinifera* (Olmo, 1942); *Citrus reticulata* (Sahar and Spiegel-Roy, 1980); *Persea americana* (Sedgley, 1981); *Zea mays* (Barnabás and Rajki, 1981); *Humulus lupulus* (Haunold and Stanwood, 1985); *Pistacia vera* (Polito and Luza, 1988b); *Carya illinoensis* (Yates and Sparks, 1989; Yates *et al.*, 1991); and *Pennisetum glaucum* (Hanna, 1990) have successfully survived storage at temperatures between 0°C and -80°C for periods longer than a year when moisture content was maintained within certain limits.

Pre-dried *Gladiolus orchidiflorus* pollen was frozen and thawed six times before its viability decreased (Koopowitz *et al.*, 1984). However, Sedgley (1981) reported that avocado pollen could only be thawed once. Therefore, it would be preferable to freeze a number of small samples rather than one large sample (Koopowitz *et al.*, 1984) to avoid repeated thawing and freezing.

2.3.2.2 Cryopreservation of pollen

Cryopreservation by freezing in liquid nitrogen at -196°C is probably the only way to inhibit metabolic functions and at the same time prevent formation of intracellular ice crystals which cause cell lysis (Mazur, 1970). The longevity of pollen under cryogenic conditions depends mainly on four factors, viz, (1) moisture content of the pollen, (2) cooling and thawing rate, (3) duration of storage, and (4) cryoprotectants (Mazur, 1970).

Pre-drying pollen is essential for successful storage in liquid nitrogen (Barnabás and Rajki, 1976; Polito and Luza, 1988b; Zhang *et al.*, 1993). The critical pollen water content is between 15 and 30% (Barnabás and Rajki, 1976; Zhang et al., 1993). Difficulties in maintaining pollen samples by cryopreservation (Crisp and Grout, 1984; Ganeshan and Alexander, 1990; Connor and Towill, 1993) normally relate to the transitions to and from freezing (cooling and thawing rate), rather than the period of time in storage (Marchant *et al.*, 1993). Maximum survival of pollen occurs at an optimum cooling rate (Ching and Ching, 1964; Tisserat *et al.*, 1983). However, many investigators have recorded good viability after direct freezing in liquid nitrogen without controlling the rate of pre-freezing (Towill, 1981; Tisserat *et al.*, 1983, Marchant *et al.*, 1993). The multistage freezing treatment itself was detrimental in the case of *Phoenix dactylifera* pollen (Tisserat *et al.*, 1983).

Cryoprotectants include polyethylene glycol, glycerol glucose and dimethyl-sulphoxide (PGD) but the lack of a cryoprotectant does not adversely affect the viability of many pollen species after cryopreservation (Tisserat *et al.*, 1983; Bowes, 1990). Glycerol as a cryoprotectant actually damages *Juglans* pollen (Luza and Polito, 1985).

Theoretically, pollen stored at -196°C should retain its viability indefinitely (Stanley and Linskens, 1974). The technique has been used successfully with a large number of crops including avocado (Sedgley, 1981); peach, almond, apricot, plum and cherry (Parfitt and Almehdi, 1984); papaya (Ganeshan, 1986); grape (Ganeshan and Alexander, 1990) and rose (Marchant *et al.* (1993). A large number of pollen samples can be compactly cryopreserved at a relatively low cost. Furthermore, pollen stored in liquid nitrogen is not subjected to loss from electrical or mechanical failure of refrigeration units (Parfitt and Almehdi, 1984).

2.3.2.3 Freeze-drying of pollen

Lyophilization or freeze-drying consists of freezing at -60°C to -80°C, followed by evacuation from 50 – 250 mm Hg to remove the water by sublimation (Stanley and Linskens, 1974). The initial moisture content of the pollen is the most important factor determining the success of this method (Ching and Ching, 1964). Therefore, it is critical that the pollen be first dehydrated (Stanley and Linskens, 1974). Pre-freezing of pollen prior to freeze-drying improved results obtained with *Pinus monticola* (Ching and Ching, 1964) and *Pistacia* (Vithanage and Alexander, 1985). Freeze-drying has the further advantage that once freeze-dried the sealed vials can be stored at room temperature (Vithanage and Alexander, 1985). Preserving pollen by freeze-drying, followed by storage at various temperatures has been found to be satisfactory in many species, including alfalfa (Hanson, 1961); coconut (Whitehead, 1962); pine (Ching and Ching, 1964); and pistachio (Vithanage and Alexander, 1985). However, *Citrus tachibana* pollen freeze-dried for 24 h did not germinate following treatment (Niedz *et al.*, 1992).

2.3.2.4 Storage in organic solvents

Storing pollen in organic solvents avoids the problem of maintaining a specific relative humidity. In addition, it may be useful for transporting pollen without refrigeration (Stanley and Linskens, 1974). However, this method of storage does not work for all organic solvents, nor for all pollen; consequently the response of pollen species to storage should be tested for each organic solvent (Stanley and Linskens, 1974). This varying response to different organic solvents may be attributed to differences in pollen anatomy and its genetic

character (Agarwal, 1983). The technique has, therefore, not yet come into general use. Agarwal (1983) tested 10 different solvents and reported that *Vitis vinifera* pollen stored in amyl alcohol and benzene showed maximum germination after six months storage. Iwanami and Nakamura (1972) studied the effect of 11 organic solvents on the pollen of *Lilium, Camellia* and *Impatiens* spp. during storage and observed that all the alcohols tested inhibited pollen tube growth in all three genera. *Citrus tachibana* pollen, stored for 24 h in anhydrous acetone, did not germinate following treatment (Niedz *et al.*, 1992).

2.3.3 *Conclusions*

1. The evidence does not warrant implicit reliance on any one temperature or any one humidity for pollen storage.

2. Storage at room temperature is not advised, even for very short periods.

3. For most binucleate pollen species, storage at extremes of humidity is fatal.

4. The higher the storage temperature, the more critical humidity control becomes.

5. Storing pollen in a deep-freezer or in liquid nitrogen can be advised as a general and practical way of storing pollen until the next season or even longer periods, provided the pollen is pre-dried before storage.

6. Because the ultimate goal for the breeder or grower using stored pollen would be to obtain seed, the effect of stored pollen on seed-set should be determined in controlled pollination studies.

2.4 Stigma receptivity, seed set, breeding systems, and interspecific crosses in the Proteaceae: a review

2.4.1 *Stigma receptivity*

All but two genera of Proteaceae are hermaphroditic (Collins and Rebelo, 1987). The flowers of most members of the Proteaceae are protandrous (Venkata Rao, 1971). Anther dehiscence occurs prior to anthesis, depositing pollen onto the tip of the style, modified for the special function of pollen presentation (Guthrie and Salter, 1950). Collins and Spice (1986) described the importance of this pollen presentation mechanism. In most Proteaceae (e.g. *Protea* and *Banksia*) the stigma is enclosed within a stigmatic cavity containing the stigma papillae (Vogts, 1971; Clifford and Sedgley, 1993). Heslop-Harrison and Shivanna (1977) surveyed 250 families, typifying the stigmas of Proteaceae as dry-papillate. Pollination occurs when pollen passes through the opening of the stigmatic cavity (stigmatic groove) to the stigma (Vogts, 1971; Ramsey and Vaughton, 1991).

The onset and duration of stigma receptivity in relation to pollen presentation and removal by pollinators, influence the reproductive success of hermaphroditic plants. There are basically two aspects of stigma receptivity in the Proteaceae: (1) the widening of the stigmatic groove, allowing pollen deposition; and (2) the secretion of stigmatic exudate for pollen germination. Various tests have been employed to test peak stigma receptivity. Stigma receptivity in the Proteaceae has been recorded by observing pollen deposition in the stigmatic cavity (groove) (Vaughton and Ramsey, 1991; Ramsey and Vaughton, 1991), by observing pollen germination on the stigma with fluorescence microscopy (Fuss and Sedgley, 1991a, 1991b), measuring the width of the stigmatic groove (Brits and Van den Berg, 1990; Fuss and Sedgley, 1991a; 1991b), by testing for non-specific esterase (Sedgley *et al.*, 1985; Collins and Spice, 1986) and by recording seed set after controlled pollinations (Brits and Van den Berg, 1990).

Most Proteaceae species investigated have flowers whose stigmatic grooves are almost closed at anthesis, and usually reach maximum width 24 – 36 hours after anthesis (Collins and Rebelo, 1987). Opening of the grooves coincides with the secretion of esterase and other

enzymes by papillae present within the grooves (Mattson *et al.*, 1974). Stigmatic grooves of *Leucospermum* have been found to open within 24 hours and attain maximum receptivity two to five days after anthesis (Brits and Van den Berg, 1990). *Banksia spinulosa* flowers attain maximum receptivity three to four days after anthesis (Vaughton and Ramsey, 1991). *B. menziesii* stigmatic grooves open 24 — 48 hours after anthesis (Ramsey and Vaughton, 1991). *B. coccinea* reaches peak stigma receptivity three days after anthesis (Fuss and Sedgley, 1991b). Collins and Spice (1986) recorded peak esterase production of *B. prionotes* at 40 hours after anthesis. Maximum pollen germination and growth of *Macadamia* (Proteaceae) pollen on the style was observed at three days post-anthesis and esterase actively reached a maximum at one to two days post-anthesis (Sedgley *et al.*, 1985). Collins and Spice (1986) working with *B. prionotes* and Fuss and Sedgley (1991a) with *B. menziesii*, observed no visible secretion on the stigmatic grooves, possibly indicating a degree of heterogeneity of stigmatic type within the family.

For effective pollination of *B. menziesii*, pollen grains must be placed within the stigmatic groove (Fuss and Sedgley, 1991a). According to these authors this can only be obtained when the groove is fully open, at three days after anthesis. This result is consistent with other studies on Proteaceae and, therefore, hand pollinations should be done on the day of peak stigma receptivity.

2.4.2 *Low seed set*

Low fruit-to-flower ratios are very common in woody perennials producing hermaphrodite flowers which exhibit self-incompatibility (Sutherland, 1986). Low seed set is extremely common in all hermaphroditic members of the Proteaceae and poses a major problem for breeding programmes and hybridization experiments (Vogts, 1960; Brits, 1984). Seed set in southern African Proteaceae was first investigated by Jordaan (1945) and Horn (1962). Horn (1962) reported very low seed set in both natural and cultivated populations of *Protea, Leucospermum* and *Serruria*. Seed set values for Australian species range from 0.1% (*Banksia chaemophyton* and *B. elegans*) to 16.3% (*Hakea erinacea*) (Barrett, 1985; cited by Collins and Rebelo, 1987). The seed set values for southern African species range from 1.1% (*Protea obtusifolia*) to c. 30.0% (*P. cynaroides*) (Vogts, 1971) and (*P. repens*) (Collins

and Rebelo, 1987). Mean fruit set for *Protea* is 5.6% according to Collins and Rebelo (1987), while Brits and Van den Berg (1990) have found it to be 20%. In contrast with this, mean fruit set for *Aulax* and *Leucadendron* is 94% and 77% respectively (Rebelo and Rourke, 1986; Collins and Rebelo, 1987).

Three major theories have been proposed to explain the low seed set observed in the Proteaceae. According to the "pollination limitation hypothesis", low seed set is due to inadequate transfer of compatible pollen, a shortage of pollen vectors, or transfer of unsuitable pollen (Whelan and Goldingay, 1986; Collins and Rebelo, 1987). The "resource limitation hypothesis" suggests that the low availability and restricted allocation of resources may contribute to low seed set (Lamont *et al.*, 1985; Lamont and Barrett, 1988; Stock *et al.*, 1989). Low seed set could be a physiologically regulated adaptive response to nutrient poor soils in southern Africa and Australia (Brits, 1992), which limit the plant resources available for the formation of seed (Lamont *et al.*, 1985). However, several lines of evidence support the view that nutrients are not responsible for the low seed set observed. Firstly, the dimensions of the follicle and infructescence in genera such as *Dryandra, Protea, Mimetes* and *Leucospermum* impose a spatial limitation on the number of seeds that can ultimately develop to maturity (Collins and Rebelo, 1987; Fuss and Sedgley, 1991b). Secondly, because of the similar plant size ranges of, for example, *Leucadendron* and *Protea*, it is difficult to accept that resources could limit seed set (Rebelo and Rourke, 1986). Collins and Rebelo (1987) concluded that there are inadequate data available to test the "resource limiting hypothesis" properly for any genus.

The "sexual selection hypothesis" is based on the sexual differences in resource allocation between the two sexual functions of the plant. "Excess" flowers would allow plants to abort seeds selectively and in so doing, increase the average genetic quality of the offspring produced (Stephenson and Winsor, 1986). Andromonoecy, where some flowers lack ovules and therefore serve only a male function, or cryptic dioecy, where one of the flower's sex organs is sterile, has also been proposed (Collins and Rebelo, 1987; Walker and Whelan, 1991). Most Proteaceae, except *Aulax* and *Leucadendron*, are functionally andromonoecious (Johnson and Briggs, 1975). However, Walker and Whelan (1991) and Clifford and Sedgley (1993) believe that andromonoecy is not a major cause of the low seed set in *Banksia* and that no direct evidence suggests that it might be widespread in the Proteaceae.

2.4.3 *Breeding systems*

It is important to understand how species reproduce because the type of pollen received (self or cross pollen) has a decisive effect on the fitness of offspring as well as on the methodology (e.g., emasculation) used in hybridization experiments. Determination of plant breeding systems is therefore fundamental to a breeding programme (Goldingay *et al.*, 1991). Despite a description of the reproductive biology of *Protea cynaroides* (L.) L. (Vogts, 1971), the breeding systems of most species of the Proteaceae are still poorly described.

The presence of pollen tubes in flowers or the formation of seeds has been used to examine the breeding system of plants (Lewis and Bell, 1981; Collins and Spice, 1986; Goldingay and Whelan, 1990). However, pollen tube data alone are inadequate for determining incompatibility because they only reflect successful pollen transfer and not necessarily fertilization and seedset (Goldingay *et al.*, 1991).

Southern African Proteaceae show variable levels of self-compatibility. *Serruria florida* (Horn, 1962) and some species of *Leucospermum* (Horn, 1962; Rourke, 1972; Brits and Van den Berg, 1990) are reported to be self-compatible, while *Protea* (Horn, 1962) and some *Leucospermum* species (Horn, 1962; Brits and Van den Berg, 1990) are self-incompatible. The same pattern of variability seems to exist in the Australian Proteaceae. A number of *Banksia* spp. have been found to be highly self-incompatible, e.g., *B. prionotes* (Collins and Spice, 1986), *B. menziesii* (Ramsey and Vaughton, 1991) and *B. spinulosa* var. *neoanglica* (Vaughton, 1988). Others, e.g., *B. serrata* (Blake, 1971), *B. paludosa* (Goldingay and Whelan, 1990) and *B. coccinea* (Fuss and Sedgley, 1991b) have been reported to be partially self-compatible. Other members of the Proteaceae, e.g., *Macadamia*, show varying levels of self-compatibility (Sedgley, 1983; Sedgley *et al.*, 1990), while others, e.g., *Grevillea barklyana*, have been found to be fully self-compatible (Ayre *et al.*, 1993).

A common problem of investigations into the breeding systems of Proteaceae, has been conclusive demonstration of self-incompatibility in a given species. A further complication is that different subspecies appear to exhibit different compatibility systems (Vaughton, 1988; Goldingay and Whelan, 1990). No detailed investigation of the genetic mechanisms controlling self-incompatibility in the Proteaceae has been done to date.

2.4.4 *Interspecific crosses*

The performances of interspecific hybrids often exceeds that of the parental species and the phenomenon is termed hybrid vigour or heterosis. Increased yield up to 100 – 150% has been recorded in *Leucospermum* 'Scarlet Ribbon' and *Leucospermum*. 'Sunrise' (Brits, 1985a; b; Sedgley and Griffin, 1989). Interspecific hybridization should not be a problem for Proteaceae species whose geographical ranges do not overlap (Lewis and Bell, 1981). However, a low incidence of interspecific hybridization has been found in natural as well as cultivated populations of Proteaceae (Rourke, 1980; Lewis and Bell, 1981; Vogts, 1982). It appears that hybridization is limited by allopatry staggered flowering times, different pollinators and/or incompatibility reactions (Lewis and Bell, 1981).

Brits (1992) estimated that 95% of all protea cultivars grown are accidentally discovered natural crosses. Fuss and Sedgley (1991a) working with *Banksia* and Brits and Van den Berg (1990) with *Protea, Leucospermum* and *Leucadendron* have developed hybridization techniques for cut-flower production. However, disappointing results have thus far been obtained with artificial hybridization, especially in the genus *Protea* (Brits, 1983). Chromosome numbers within Proteaceae genera are constant (De Vos, 1943). Systematic, controlled breeding towards definite goals is essential for developing the full potential of proteas (Brits, 1992). Because physiologically regulated incompatibility isolation mechanisms are generally more easily overcome than chromosomal isolation mechanisms, the prognosis for overcoming interspecific incompatibility in proteas appears promising (Brits, 1992).

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CHAPTER 3

FACTORS AFFECTING *IN VITRO* GERMINATION OF *PROTEA* POLLEN

Abstract: The effect of pH, sucrose, boric acid and temperature upon *in vitro* germination of *Protea repens* (L.) L. cv. 'Embers' pollen was investigated in hanging-drop culture in order to establish an optimum medium for germination. The basal medium consisted of 300 mg.ℓ^{-1} Ca $(NO_3)_2.4H_2O$; 200 mg.ℓ^{-1} $MgSO_4.7H_2O$ and 100 mg.ℓ^{-1} KNO_3 in distilled water. Optimum levels of the variables tested for fresh *Protea* pollen were found to be within ranges pH 5 – pH 8, 0.4 – 0.7 M sucrose concentration, 50 – 500 mg.ℓ^{-1} boric acid concentration and 5 – 30°C incubation temperature. In all cases pollen tubes did not reach a length exceeding 120 μm.

3.1 Introduction

The germination of the pollen of a wide range of species has been studied in artificial media, but no satisfactory *in vitro* pollen germination technique has been described for *Protea*. It is essential to have a reliable technique of determining pollen quality in order to evaluate pollen to be used in cross-pollination programmes and to evaluate the suitability of storage methods in preserving pollen. The development of a suitable pollen germination medium has therefore been a matter of high priority in the *Protea* breeding programme.

The viability of fresh and stored pollen is best determined by germination tests (Heslop-Harrison *et al.*, 1984) but this depends critically on the ability of the investigator to formulate a suitable medium. Proteaceae pollen is recorded as binucleate (Davis, 1966), and can be cultured in sucrose solutions (Garside, 1946). Horn (1962) germinated *Protea* pollen in 25% sucrose and 50 mg.ℓ^{-1} boric acid, and obtained a maximum germination of 86%. The viability of *Protea* pollen can be effectively tested by germinating in 12% sucrose plus 100 mg.ℓ^{-1} boron (Brits and Van den Berg, 1990). Shchori *et al.* (1992) tested several Proteaceae species including *Banksia, Protea* and *Leucospermum* in a series of liquid sugar media, including Tailor's medium (Tailor, 1972). They concluded that Proteaceae pollen should be tested immersed in Tailor's medium in a hanging-drop. In preliminary investigations (Van der Walt & Littlejohn, in press), the Brewbaker and Kwack (1963) medium resulted in excellent germination of fresh *Protea* pollen (> 90%), although pollen tubes did not reach a length of more than 120 μm before bursting.

The present study was undertaken to develop a reliable germination medium for *Protea* pollen. *Protea repens* cv. 'Embers' was the most readily available pollen source and was consequently used in this study.

3.2 Material and Methods

3.2.1 *Pollen collection*

All experiments were conducted during 1993 on pollen of a single clone of *Protea repens* (L.) L. cv. 'Embers', planted in an experimental plantation at Elsenburg (latitude 33°51'S, longitude 18°50'E; 177 m a.s.l.) in South Africa. Prior to the experimental period the plantation had been subjected to routine management practices, including drip-irrigation during summer months. Harvesting of blooms in previous years served as the only form of pruning of the bushes. Five inflorescences each from five different plants, with approximately one half of the florets having undergone anthesis, were harvested and brought to the laboratory where the stems were placed in water. All the open florets were removed and 16 hours later all florets that had subsequently opened were harvested (c. 25 per inflorescence). The pollen was scraped off and thoroughly mixed. The pollen mixture was immediately used to provide five samples for each experimental treatment in the germination experiments which followed.

3.2.2 *Pollen germination*

Four independent experiments were carried out to determine optimum factor levels for pollen germination percentage. *In vitro* germination of pollen was carried out over a period of five weeks, commencing in the middle of the flowering season of the cultivar. The capacity of the pollen to germinate and to produce normal pollen tubes was tested by the hanging-drop technique of Van Tieghem (1869) using an artificial sucrose growth medium. Preliminary germination tests established no significant effects of $Ca(NO_3)_2.4H_2O$, $MgSO_4.7H_2O$ and KNO_3 concentrations on germination percentages or tube growth of *Protea* pollen, so these were not included as variables in the experiments. The variables were pH level (from 2 to 11), sucrose concentration (from 0 to 1.0 M), boric acid (H_3BO_3) level (from 0 to 1 000 mg.ℓ^{-1}) and incubation temperature (from 5° to 40°C). The basal medium (Brewbaker and Kwack, 1963) consisted of 300 mg.ℓ^{-1} Ca $(NO_3)_2.4H_2O$; 200 mg.ℓ^{-1} Mg $SO_4.7H_2O$; and 100 mg.ℓ^{-1} KNO_3 in distilled water. In all experiments except that involving sucrose concentration as variable, the sucrose concentration was adjusted to 0.5 M, and in

all experiments except that involving pH as variable, the pH was adjusted to 7.0 using 0.1 M HCl or KOH solutions. Since germination occurred within a short period of time, it was scored after three hours under an Olympus BH-2 light microscope at a magnification of x 200. A minimum of 200 randomly selected pollen grains in four different fields (c. 50 per field) were scored for germination. Only pollen grains producing tubes longer than the grain diameter were scored as having germinated.

3.2.3 *Statistical procedures*

The data were analysed using regression analysis. For the experiments with pH and sucrose as variables, second order polynomials were fitted to the data. For the experiments with boric acid and temperature as variables, the slopes of the two distinct linear trends and their point of intersection were used to obtain the optimum levels (Draper & Smith, 1966).

3.3 Results

Germination of the pollen commenced within 15 minutes after inoculation and was completed within two hours, after which the pollen-tube ends ruptured and the contents expelled (Fig. 3.1). Pollen tubes germinated from only one aperture. In no experiment did pollen tubes exceed a length of 120 μm (Fig. 3.1). The germination percentage increased with increasing pH levels up to pH 6.4, after which it decreased (Fig. 3.2). The pollen germination percentage at the optimum pH (6.4) was 86% ± 10.1. However, variation in pH level between 5.0 and 8.0 had little effect on pollen germination. A pH value of 7.0 was therefore used in the experiments which followed.

A similar trend was observed in the experiment with sucrose concentration as variable. A germination percentage of 97% ± 3.4 was obtained at the optimum of 0.6 M (Fig. 3.3). Variation in sucrose concentrations between 0.4 M and 0.7 M had little effect on pollen germination but extreme sucrose levels inhibited germination as well as pollen tube growth. A sucrose concentration of 0.5 M was therefore used in subsequent experiments.

In the experiments with boric acid and temperature as variables two distinct linear trends were evident. In the case of boric acid a significant amount of pollen (32.8%) germinated with boric acid absent in the medium, but the majority of pollen grains formed only short protuberances and were therefore not scored as having germinated.

The point of intersection of the two linear trends fitted to the data for boric acid was 53 ± 3.1 mg.ℓ^{-1}. Pollen germination increased linearly (slope = 1.2868, $P < 0.01$) with increasing boric acid concentration up to this point, after which it decreased linearly (slope = -0.0329, $P < 0.01$) with further increase in the boric acid concentration (Fig. 3.4). Boric acid levels between 50 mg.ℓ^{-1} and 500 mg.ℓ^{-1} appear to have little effect on the maximum pollen germination (100%). Boric acid at 100 mg.ℓ^{-1} was therefore used in the experiments which followed. The point of intersection of the two linear trends fitted to the data of the experiment with temperature as variable was 29 ± 0.6°C (Fig. 3.5). Pollen germination remained almost constant (slope = 0.1294, $P = 0.6443$) with increasing temperature up to this point after which it decreased sharply (slope = -9.5775, $P < 0.01$). An incubation temperature of 25°C was used in subsequent experiments.

3.4 Discussion

Although a complete study of all possible factors influencing pollen germination was not made, the high germinabilities (> 90%) observed in these experiments suggest that the medium defined is likely to be as close to optimum as would be required in any practical application despite the short pollen tubes observed. *Protea* pollen grains are relatively small (c. 30 μm in diameter); it is possible that the pollen grain's own resources to produce and sustain pollen tube growth might be very limited and that the pollen might need additional growth factors from the style for it to produce longer tubes. This needs to be investigate further.

Jojoba pollen germinated poorly at a temperature lower than 15°C (Lee *et al.*, 1985). *Protea* pollen showed no such tendency although it took slightly longer to germinate and pollen tube growth was a little slower. Pollen germination was maximal over a wide incubation temperature range. Since Schmucker (1935) demonstrated the essential role of

boric acid on pollen germination, many investigators (Visser, 1955; Brewbaker and Kwack, 1963; Vasil, 1964; De Bruyn, 1966b; Dickinson, 1978) have confirmed this for various pollen species. This study showed that a small amount of boric acid is essential for optimal pollen germination in *Protea*.

Considering all the information obtained in the present study, the following medium was estimated as optimal for *Protea* pollen: 300 mg.ℓ^{-1} $Ca(NO_3)_2.4H_2O$; 200 mg.ℓ^{-1} $MgSO_4.7H_2O$; 100 mg.ℓ^{-1} KNO_3; 100 mg.ℓ^{-1} H_3BO_3; 0.5 M sucrose; pH 7.0; and an incubation temperature of 25°C.

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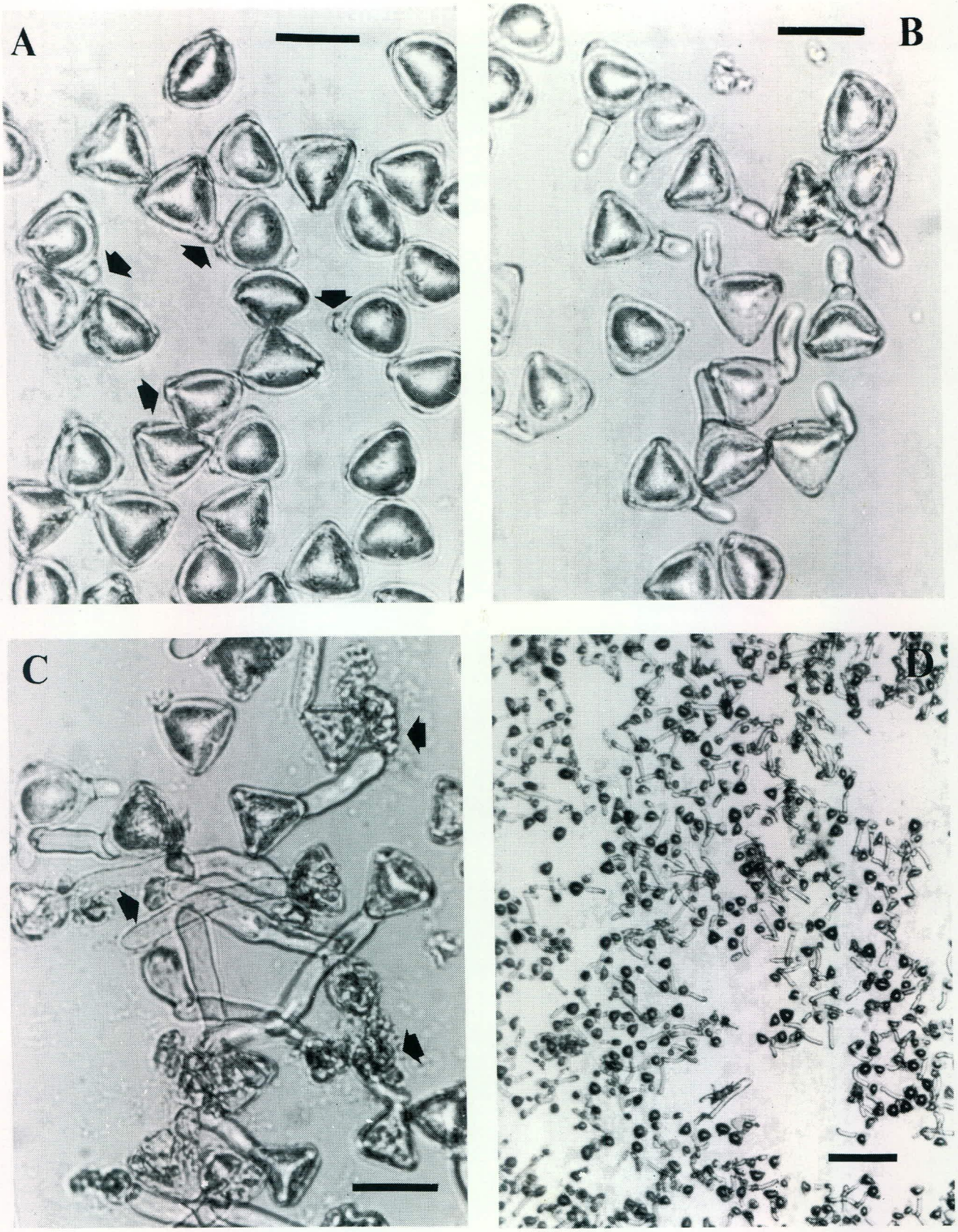

Fig. 3.1 Light micrographs of *P. repens* cv. 'Embers' pollen germination and tube growth. A. Pollen showing early pollen tube emergence (arrows) 15 min. after inoculation. x 400 (bar = 30 μm). B. Pollen tube growth 30 min. after inoculation. x 400 (bar = 30 μm). C. Pollen tube growth one hour after inoculation. Some pollen tubes have already ruptured and the contents expelled (arrows). x 400 (bar = 30 μm). D. Germinating pollen grains showing sowing density. x 100 (bar = 100 μm).

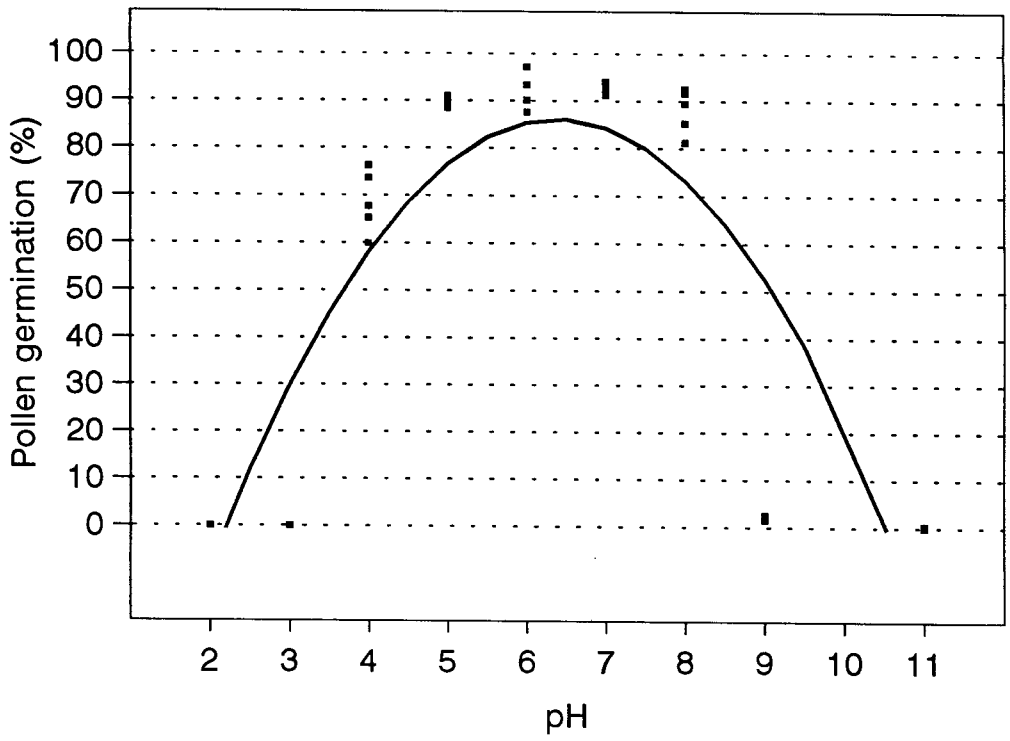

Fig. 3.2 The effect of pH of the medium on *in vitro* germination of *P. repens* cv.'Embers' pollen. The relationship between germination percentage and pH is described by the equation $y=-114.3399+62.750\ x-4.9118x^2$; $r^2=0.7294$; $P<0.01$.

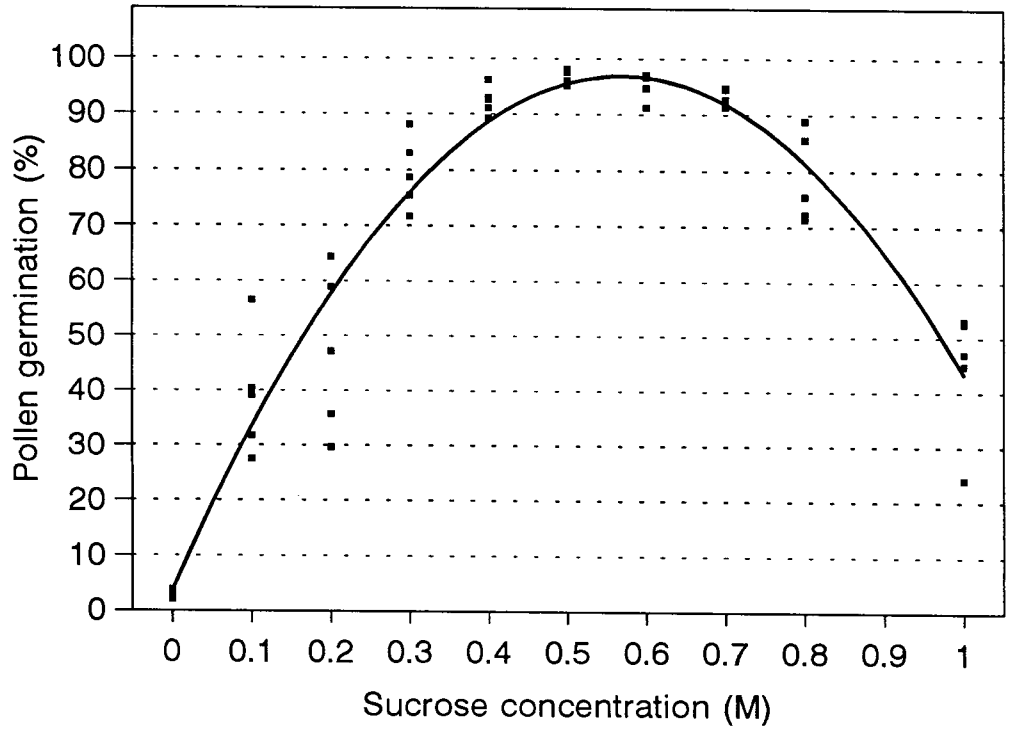

Fig. 3.3 The effect of sucrose concentration of the medium on *in vitro* germination of *P. repens* cv. 'Embers' pollen. The relationship between germination percentage and sucrose concentration is described by the equation $y=3.6968+327.7215x-288.1241x^2$; $r^2=0.9302$; $P<0.01$.

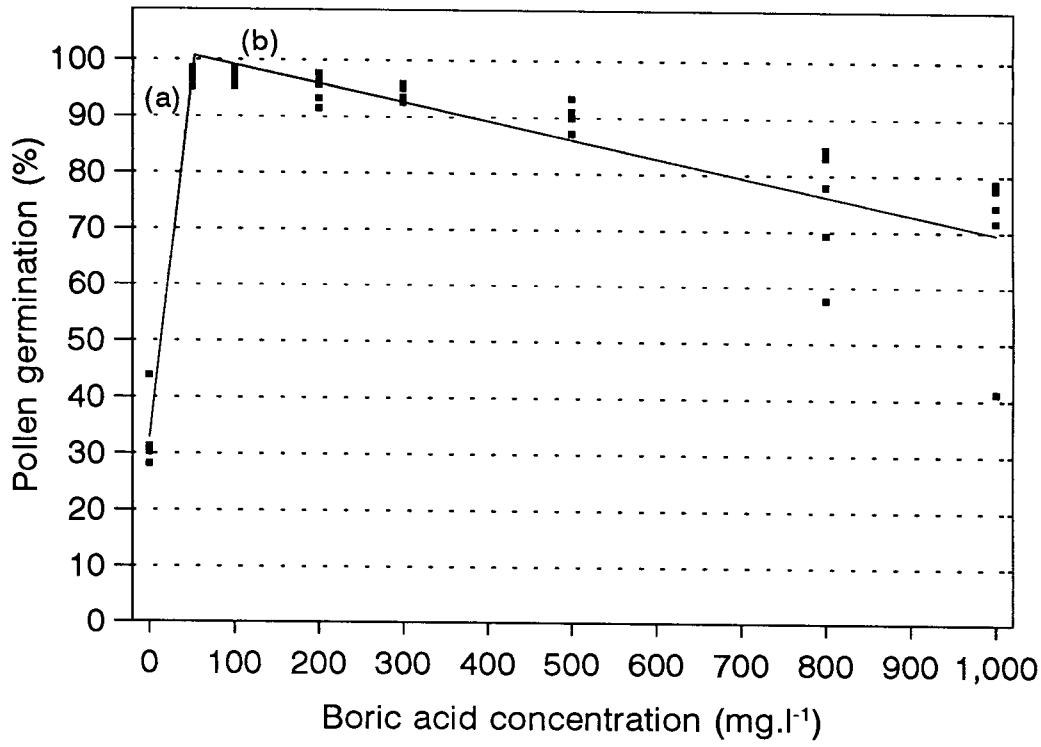

Fig. 3.4 The effect of boric acid concentration of the medium on *in vitro* germination of *P. repens* cv. 'Embers' pollen. The relationship between germination percentage and boric acid concentration is described by the following equations: (a) $y=32.8124+1.2868x$ (b) $y=102.4573-0.0329x$; $r^2=0.9935$; $P<0.01$.

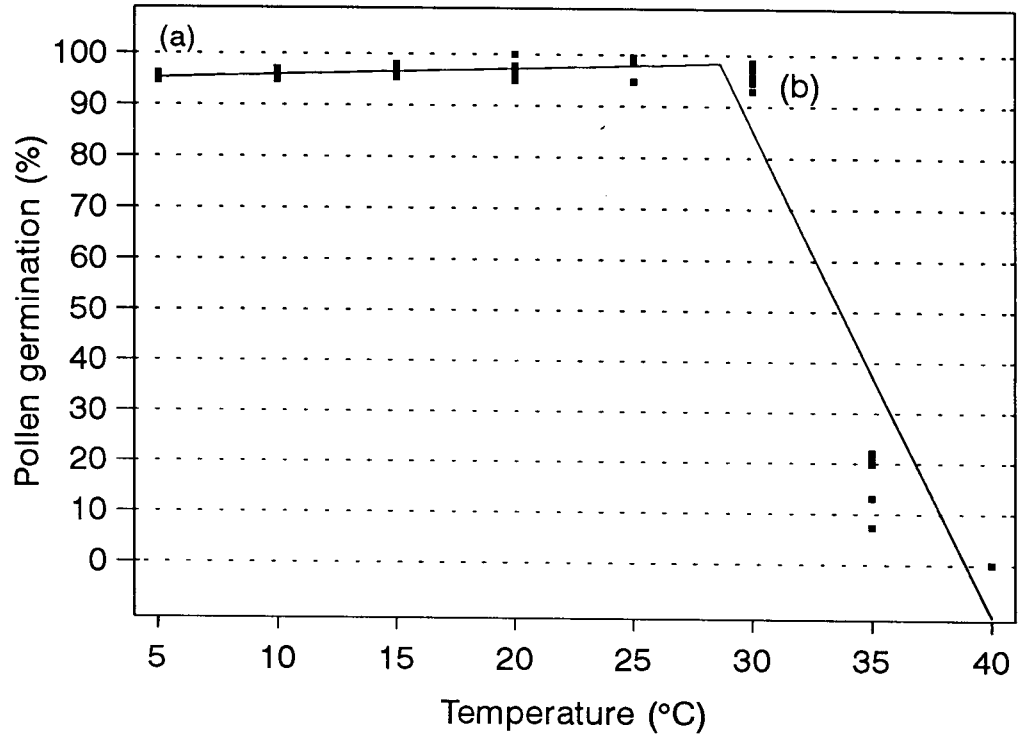

Fig. 3.5 The effect of incubation temperature of the medium on *in vitro* germination of *P. repens* cv. 'Embers' pollen. The relationship between germination percentage and incubation temperature is described by the following equations: (a) $y=94.6197+0.1294x$ (b) $y=372.6840-9.5775x$; $r^2=0.9876$; $P<0.01$.

CHAPTER 4

STORAGE AND VIABILITY TESTING OF *PROTEA* POLLEN

Abstract: The influence of storage temperature and humidity on pollen viability was studied in four *Protea* clones. Pollen was stored at a range of temperatures and relative humidities for up to one year and tested for ability to germinate *in vitro*. Pollen of *P. repens* cv. 'Sneyd', *P. eximia* cv. 'Fiery Duchess' and *P. magnifica* clone 'T 84 07 05' stored in liquid nitrogen (-196°C) and in a freezer (-14° to -18°C) retained a germination percentage as high as that of fresh pollen regardless of humidity. Humidity control became increasingly important at storage temperatures above 0°C. The study showed that long-term storage of protea pollen is not feasible at temperatures above 0°C. The relationship between germinability and fluorochromasia (FCR) was studied during storage of cv. 'Sneyd' pollen. The correlations between FCR and germinability were found to be low and nonsignificant. Fifteen month old cryopreserved cv. 'Sneyd' pollen was shown to retain its ability for fertilization and seed set equal to that of fresh pollen. 'Sneyd', 'Fiery Duchess' and 'T 84 07 05' pollen could be repeatedly thawed and frozen in liquid nitrogen before its germinability *in vitro* decreased.

4.1 Introduction

Favourable conditions for pollen storage have been investigated for many agronomic and horticultural crops (Lee *et al.*, 1985; Yates *et al.*, 1991). In general, low temperature and low relative humidity maintain viability, but there are numerous exceptions (Stanley and Linskens, 1974). Genetic improvement of *Protea* by breeding and selection is gaining momentum in a number of countries around the world but there is only limited information available regarding suitable pollen storage conditions. Knowledge concerning pollen storage is an important aid in hybridization programmes because it facilitates the crossing of species with differing flowering periods. It may also have potential for long-term germplasm storage, especially of unique novel genotypes.

Shchori *et al.* (1992) have found that pollen of species of Proteaceae have higher viability at 5°C storage than at deep-freeze storage and that pollen viability may be maintained to a reasonable degree at 5°C for at least one month. Fresh *Protea* pollen has been found viable for up to six days of storage at room temperature and up to six weeks at 5°C (Brits and Van den Berg, 1990). *Banksia menziesii* pollen viability has been found to decrease rapidly at ambient temperature and most pollen was inviable after 24 hours (Ramsey and Vaughton, 1991). Preliminary investigations have found that *Protea repens* cv. 'Sneyd' pollen has high viability (up to 70%) after seven days and decreases to 18% after nine days in the field. On the other hand, it would be of practical value to be able to store pollen for long periods at approximately -20°C to allow the use of conventional and readily available freezers for storage (Pinney and Polito, 1990). The purpose of this study was therefore to develop a practical method of storing *Protea* pollen for a year or more with sufficient viability to be used in controlled hybridizations using inexpensive, commonly available supplies and equipment.

Since the ultimate goal for a breeder using stored pollen is to obtain viable seeds, the ability of stored *Protea* pollen to effect seed set in controlled pollinations in the field is also investigated in this study, as well as the number of times pollen can be frozen and thawed without serious loss of viability. *Protea* species used in this study all have commercial value and are readily available in experimental plantations.

4.2 Material and Methods

4.2.1 *Pollen collection*

Experiments were conducted from 1992 to 1994 on four clones planted in experimental plantations at Elsenburg (latitude 33°51'S, longitude 18°50'E; 177 m.a.s.l.) and Riviersonderend (latitude 34°08'S, longitude 19°54'E; 168 m.a.s.l.) in South Africa. Prior to the experimental period all plants had been subjected to routine plantation management practices, including drip-irrigation during the summer months. Harvesting of blooms in previous years served as the only form of pruning of the bushes. For the storage experiments, pollen of *Protea magnifica* Link. clone T 84 07 05, *Protea eximia* (Salisb. ex Knight) Fourcade cv. 'Fiery Duchess', *Protea repens* (L.) L. cv. 'Sneyd', and *Protea aristata* Phill cv. 'Aristocrat' was used. Five inflorescences per plant from five plants were used and handled in the same way as previously described (Chapter 3), with the exception that in this case the pollen from each plant (five inflorescenses) was mixed and used as a single replicate. All replicates were first dried for 24 hours in a desiccator over silica gel (blue) at 5°C, after which the pollen mixtures were divided into separate samples and placed in gelatine capsules for storage. The number of samples was large enough to ensure that each sample would be tested only once.

4.2.2 *Pollen storage*

The gelatine capsules with pre-dried pollen samples were stored at room temperature (22° to 27°C), in a household refrigerator (2° to 7°C), in a household freezer (-14° to -18°C) and in liquid nitrogen (-196°C). At all the temperatures, except liquid nitrogen which has negligible water vapour pressure, the pollen samples were stored at relative humidities (RH) of 10, 30 and 60%. The humidities were maintained by means of different concentrations of sulphuric acid according to prescriptions of Solomon (1951) and Weast (1988). The atmospheres were produced in closed 1 000 mℓ flasks and were allowed to equilibrate for at least one month before use. The freezing points of solutions were below the temperatures to which they were exposed in the freezer. Pollen of *P. repens* cv. 'Sneyd' was stored at an additional humidity of 0%, maintained over silica gel (blue) in a desiccator. For storage

at -196°C, the pre-dried pollen samples were individually placed in 5 cm^3 plastic cryovials which were directly submerged in liquid nitrogen in a nitrogen storage vessel. No pre-cooling or cryoprotectant was used to treat pollen before freezing.

4.2.3 *Viability tests*

4.2.3.1 *In vitro* germination

As a control on storage procedures, each replicate was scored for pollen germinability before storage. Stored samples were thawed and rehydrated for three hours at 25°C in a 100% RH atmosphere chamber before testing. Pollen stored at room temperature was tested for germinability every 15 days while the other treatments were tested after 30, 90, 180, 270 and 360 days of storage. The germination medium used in the storage experiments consisted of 0.4 M sucrose, 100 mg.ℓ^{-1} H_3BO_3, 300 mg.ℓ^{-1} $Ca(NO_3)_2.4H_2O$, 200 mg.ℓ^{-1} $MgSO_4.7H_2O$, 100 mg.ℓ^{-1} KNO_3 in distilled water. The pH was adjusted to 7.0 using 0.1 M HCl or KOH solutions. Pollen germination percentages were determined by the procedure previously described (Chapter 3).

4.2.3.2 Fluorometry

A comparison was made between the *in vitro* germination test and the fluorochromatic (FCR) test. Only stored pollen of *P. repens* cv. 'Sneyd' was used for this purpose. The fluorochromatic (FCR) test procedure of Greissl (1989), using fluorescein diacetate/ Propidium-iodide, was used. As a control, each pollen replicate was tested before storage with the FCR test procedure. Each stored pollen sample was divided into two sub-samples , one being assayed for germination and the other using the FCR procedure at each of the testing times.. Pollen grain fluorescence was determined with a Nikon Biophot microscope equipped with an episcopic-fluorescence attachment and a B-2A filter system consisting of a dichroic mirror (510 nm), a blue excitation filter (450 — 490 nm), and a barrier filter (520 nm). All pollen grains which fluoresced brightly were scored as viable. Viability percentages were determined,using five replicates of approximately 200 grains each.

4.2.3.3 Pollination

Viability of 15-month-old cv. 'Sneyd' pollen, stored in liquid nitrogen, was further tested by pollination in the field, and the results compared with those obtained with fresh pollen. The stored pollen was frozen and thawed once before this experiment. Thirty *P. repens* cv. 'Sneyd' inflorescences, with approximately one half of the florets having undergone anthesis, were labelled and all open florets removed with scissors (Fuss and Sedgley, 1991a). The inflorescences were covered with semi-rigid wire mesh cages (2 x 2 mm apertures) to exclude pollinators (Coetzee and Giliomee, 1985) and to prevent contact between the florets and the pollination bag (Fuss and Sedgley, 1991a) (Fig. 4.1). After 16 hours the cages were removed and all remaining unopened florets were removed, leaving a ring of between 15 and 35 open florets (Fig. 4.1C). Self-pollen was not removed from these florets. The inflorescences were recaged until pollination at three days after anthesis. Ten inflorescences were pollinated with fresh cv. 'Sneyd' pollen (self-pollinated), ten inflorescences were pollinated with 15-month-old liquid nitrogen-stored pollen, and ten inflorescences were left unpollinated as controls. Florets were pollinated in the morning by touching their stigmas with pollen presenters from newly opened donor florets which had also been caged to prevent contamination or by touching stigmas with 15-month old liquid nitrogen-stored pollen. The stored pollen had been rehydrated before pollination, as previously described.

Three replicates (nine inflorescences) were harvested seven days after pollination, when penetration of the ovule had occurred. The bottom part of the pollinated pistils, including the ovary, were carefully dissected out of the involucral receptacles, fixed in Carnoy's solution for 24 h and transferred to 70% ethanol for storage at room temperature. Before processing ovaries for fluorescence microscopy (Martin, 1959; Kho and Baër, 1968), ovaries were transferred to 30% ethanol, washed in distilled water (3 x), softened for 30 min. in 0.8 N sodium hydroxide at 75°C, washed again in distilled water (x 3), and stained overnight in a 0.1% solution of water-soluble aniline blue dye in 0.1 N $K_3PO_4.H_2O$. For observation of pollen tubes the ovaries were placed on a slide and the cover slip was gently pressed to spread the material. Fluorescing pollen tubes were observed by means of a Nikon Biophot microscope, equipped with a episcopic-fluorescence attachment and a UV-2A filter system consisting of a dichroic mirror (430 nm), an ultraviolet excitation filter (380 – 425 nm), and

a barrier filter (450 nm). The number of pollen tubes in the ovary was recorded as well as the number of tubes that had penetrated the ovules.

Seven replicates (21 inflorescences) were harvested seven months after pollination, when the achenes had matured (Van Staden, 1978), to record seed set for all the flowers pollinated previously. The infructescences were dried and the number of seeds per inflorescence was recorded by dissecting the pollinated one-seeded achenes with a scalpel. The percentage seedset of pollinated florets per seed head was calculated.

4.2.4 *Number of freeze/thaws*

Liquid nitrogen (LN_2) stored pollen, previously used for the storage experiments, was used for this experiment. The pollen samples were previously stored in LN_2 for varying periods: *P. magnifica* clone 'T 84 07 05' for 5 months; *P. eximia* cv. 'Fiery Duchess' for 3½ months; and *P. repens* cv. 'Sneyd' for 22 days. All pollen samples were frozen and thawed once before commencing with this experiment. Each cycle of freezing and thawing involved the following procedure: (1) Pollen samples were removed out of LN_2 storage and thawed and rehydrated at 25°C in a 100% RH atmosphere chamber for three hours before testing *in vitro* germinability as described previously. (2) After testing, the pollen samples were dried for 24 hours in a desiccator over silica gel (blue) in a refrigerator at 5°C. (3) After drying, the pollen samples were plunged directly back into the LN_2 for at least 24 hours before starting the the next cycle of testing. This procedure was repeated for nine cycles. Five replicates per pollen species were used.

4.2.5 *Statistical analysis*

The four clones in the storage experiments were investigated in separate experiments. Each experiment consisted of five replications in randomized blocks with a three-factor factorial design for treatments.

The fluorometry experiment consisted of five randomized blocks with a split-plot design for treatments. Pearson product-moment correlations were calculated to measure the strength

of the linear relationship between FCR percentage and germinability for each storage treatment.

The pollination experiment consisted of randomized blocks. Analysis of variance (ANOVA) was performed for each part of the experiment separately and Pearson product-moment correlations were calculated to measure the strength of the linear relationship between different variables connected with pollen tube growth.

The freeze/thaw experiment consisted of randomized blocks. Linear regression analysis was performed on data from the storage experiment for each of the clones to determine whether storage time in liquid nitrogen affected the *in vitro* germinability of pollen. A separate ANOVA was performed after the data were subjected to a working logit transformation for each of the three clones. The test of homogeneity of residual variances gave a non-significant chi-square value indicating comparable accuracy of experiment (John and Quenouille, 1977). One overall ANOVA was then performed.

For all experiments, ANOVA was performed using SAS statistical software version 6.08 (SAS Institute Inc., Cary, NC, USA). Student's least significant differences (LSD) were calculated at the 5% level of probability to compare treatment means. For all other effects in the ANOVA a probability level of 5% was considered as significant.

4.3 Results

4.3.1 In vitro *germination*

Results of the *in vitro* germination tests that were carried out at intervals while the pollen was in storage in the four experiments are shown in Figures 4.2 to 4.5. For each of the experiments, analysis of variance showed a significant three-factor interaction ($P < 0.01$) between method of pollen storage, relative humidity and storage time of the pollen. Therefore, the main effects of the analysis could not be interpreted independently. All four clones tested showed a very high (> 80%) initial (fresh) germination percentage. Three of the clones tested gave similar results, while one clone, 'Aristocrat', gave significantly lower germination percentages over many of the treatments.

From Figures 4.2 to 4.5 it can be seen that the RH at which the pollen was stored, had a significant influence on the pollen germinability of all four clones stored at room temperature (22°C to 27°C). Germinability was maintained longest at 30% RH while storage at 10 and 0% RH gave less favourable results. Viability was completely lost in all four clones within 30 days at 60% RH. At the most favourable humidity (30%) germination remained at more than 60% for only 15 days, and was completely lost within 90 days for all four clones.

It appears from Figures 4.2 to 4.5 that the influence of the RH on the germinability of stored pollen in a fridge (2° to 7°C) was qualitatively the same as at room temperature except that pollen stored at 2° to 7°C maintained its germinability at any RH roughly three times as long as at 22° to 27°C. At the most favourable humidity (30%) the germination percentage was more than 60% after variable lengths of time depending on clone; 'Sneyd': 180 days; 'Fiery Duchess' and 'T84 07 05': 90 days; and 'Aristocrat': 30 days. Germinability at this RH was completely lost for three of the clones within 270 days while 'Sneyd' pollen still had some germinability (17.5%) after 360 days (Fig. 4.4B). Germinability of all four clones at the 60% storage treatments, was completely lost within 180 days.

In the freezer (-14° to -18°C) storage treatment (Figures 4.2 to 4.5), cv. 'Sneyd', cv. 'Fiery Duchess' and clone 'T84 07 05' germinated as well as fresh pollen after 360 days of storage, regardless of humidity, with only the 60% RH treatment of cv. 'Sneyd' being significantly lower than that of fresh pollen. In contrast, pollen of cv. 'Aristocrat' showed a significant decline in germinability (50 — 70%) over the period of 360 days (Fig. 4.2C). Moreover, differences also developed between the RH treatments for cv. 'Aristocrat', with the 10 and 30% RH treatments significantly better than the 60% RH after 360 days of storage. In addition, pollen of cv. 'Aristocrat' showed a significant increase in germinability at 270 days compared to 90 and 180 days in the 10 and 30% RH treatments; the reason for this is unclear.

Results for the liquid nitrogen (-196°C) treatments are given in Figures 4.2 to 4.5. Only cv. 'Aristocrat' pollen showed a significant decline (25%) in germinability after 360 days of

storage. An unexplained increase in germinability at 270 days compared to 90 and 180 days again occurred for this clone (Fig. 4.2D).

4.3.2 *Fluorometry*

The fluorescence of pollen grains stained with the contrast-staining technique (fluorescein-diacetate/Propidium-iodide) varied from bright yellow to reddish-brown (Fig. 4.6). Only reddish-brown grains were classified as non-viable. The relationship between the FCR and germinability of pollen stored at different humidities and temperatures are shown in Figures 4.7 and 4.8. Analysis of variance showed a significant four-factor interaction ($P < 0.01$) between method of pollen storage, relative humidity, the time pollen was stored and method of testing. Therefore, the main effects of the analysis could again not be interpreted independently. For each storage treatment, correlations between FCR percentage and germinability are given in Table 4.1. Correlation coefficients (r) were low and non-significant, except for the 60% RH fridge treatment with $r = 0.89$ ($P < 0.05$).

From Figure 4.7B it can be seen that the RH at which the pollen was stored had, as in the germination test, a significant influence on the FCR percentage of pollen stored at room temperature (22° to 27°C). FCR capacity was retained longest at 10% RH while storage at 0% and 10% RH gave slightly less favourable results. As for viability as measured by germinability (Fig. 4.7A), FCR capacity was completely lost within 30 days under 60% RH (Fig. 4.7B).

Contrary to the results obtained with the germination test with pollen stored in the fridge, the FCR test at this temperature indicated no significant differences or loss in viability for the different RH treatments, except at 60% RH, after 360 days (Fig. 4.7B). At 60% RH the pollen retained a FCR percentage exceeding 60% for only 120 days and was completely inviable after 270 days.

The FCR test further indicated that cv. 'Sneyd' pollen retained a FCR percentage as high as that of fresh pollen after 360 days of storage, regardless of humidity, in the freezer as well as in liquid nitrogen (Fig. 3.8B).

4.3.3 *Pollination*

Controlled hand self-pollination of 'Sneyd' florets with pollen cryopreserved for 15 months resulted in a normal seed set (80.2%), comparable to that derived from pollination with fresh pollen (75.3%) (Fig. 4.9). Moreover, no significant differences were observed between cryopreserved and fresh pollen with regard to the percentage of florets containing pollen tubes, the percentage of florets with ovule penetration, and the mean number of pollen tubes per floret (Fig. 4.9). The control (unpollinated) differed highly significantly from the two hand-pollination treatments in all four of the variables tested. Correlation analysis between the three pollen tube variables indicated that correlation coefficients (r) were strong and highly significant ($P < 0.01$).

4.3.4 *Number of freeze/thaws*

Analysis of variance showed a significant two-factor interaction ($P < 0.01$) between clone and number of freeze/thaw cycles. Therefore the main effects of the analysis could again not be interpreted independently. Germinability comparable to that of fresh pollen could be maintained for seven cycles in cv. 'Sneyd', six cycles in clone 'T84 07 05' and only for four cycles in cv. 'Fiery Duchess' (Fig. 4.10). Further freeze/thawing caused pollen germination percentages of all three clones to decline significantly. After nine cycles, pollen of both cv. 'Sneyd' and clone 'T84 07 05' still germinated at more than 60%, while that of cv. 'Fiery Duchess' had declined significantly (39.8%) (Fig. 4.10).

4.4 Discussion

This study indicated that storage of *Protea* pollen above 0°C may be adequate in the very short-term but definitely not over the long-term in agreement with results of numerous studies on various other species (Ganeshan, 1985; Polito and Luza, 1988b; Yates *et al.*, 1991). Viability at temperatures above 0°C declines rapidly, especially when the humidity of the storage atmosphere is not controlled (Luza and Polito, 1985).

Controlling humidity at temperatures below 0°C has a marked effect on the retention of germinability of pollen (Allan, 1963; Luza and Polito, 1985; Polito and Luza, 1988; Hanna, 1990). However, this study has shown that for many *Protea* species, controlling humidity below 0°C had no advantage in agreement with results of Visser (1955) working with pear, apple, tomato and azalea pollen. Therefore, it may be possible to store many, if not all, *Protea* pollen relatively cheaply in an ordinary household freezer without humidity control for periods long enough to suit breeding requirements. Furthermore, a satisfactory cryostorage method to preserve *Protea* pollen was obtained through direct immersion into liquid nitrogen. Theoretically, pollen stored in liquid nitrogen should retain its viability indefinitely (Stanley and Linskens, 1974), provided precautions are taken to reduce pollen moisture before storage (Yates *et al.*, 1991). The poor results obtained in this study with cv. 'Aristocrat' over all the storage treatments might make extrapolation of pollen longevity from one *Protea* species/clone to another inappropriate. Differences in pollen longevity among cultivars is known to occur (Parfitt and Almehdi, 1984). Therefore, more *Protea* species/clones should be tested before the results of this study can be applied to the whole genus. In view of the results of this study, it can be concluded that freezer and liquid nitrogen storage can now be successfully employed for the long-term storage of *Protea* pollen to assist breeding programmes.

The finding that the FCR test does not correlate well with the *in vitro* germination test indicates that this assay is not suitable for viability determinations of stored *Protea* pollen. Similar results have been reported by Widrlechner *et al.* (1983) for azaleas. Heslop-Harrison *et al.* (1984) also experienced problems when the method was used on stored pollen. However, many other studies (e.g. Jansson and Warrington, 1988; Griessl, 1989; Radicati Di Brozolo *et al.* 1990; Pinney and Polito, 1990) have shown that the FCR test provided an excellent guide to pollen germinability in a number of different species. Inspection of the graphs indicates that germinability scores are always lower than the FCR scores except where they lie within the margins of error. According to Heslop-Harrison and Heslop-Harrison (1970) this is to be expected because a pollen grain showing FCR has at least the potential for germination, whereas one that does not certainly lacks this. It can be concluded that the *in vitro* germination test is the most accurate and routine method for determining viability of *Protea* pollen held in long-term storage.

Several investigators (e.g. Griggs *et al.* 1971; Sedgley, 1981; Ganeshan and Alexander, 1990) have shown that even though pollen is able to germinate *in vitro*, fertilization and seed production may fail. However, the ability of *Protea* pollen to effect fertilization and produce seed does not appear to be influenced by cryopreservation. Similar results have been obtained with cryogenically stored pollen of jojoba (Lee *et al.*, 1985); papaya (Ganeshan, 1986); pecan (Yates and Sparks, 1990); narcissus (Bowes, 1990); and rose (Marchant *et al.*, 1993). Whether or not seed set can be obtained with *Protea* pollen stored at higher temperatures, requires further investigation. Seed produced by cryopreserved broccoli pollen germinated poorly (Crisp and Grout, 1984) and should be investigated for *Protea* pollen as well.

Avocado pollen can only be thawed once and looses viability when refrozen (Sedgley, 1981). However, the present study has indicated that *Protea* pollen can be frozen/thawed at least seven times with sufficient retention of germinability ($> 60\%$), which is considered to be the minimum acceptable level for breeding purposes (Visser, 1995). This results is in agreement with those obtained by Koopowitz (1984) with gladiolus pollen stored at -40°C. The period of storage of pollen of the three clones in liquid nitrogen before commencing the experiment had no influence on the number of freeze/thaw cycles before germinability declined. This might have been expected since results of the storage experiments and linear regression analysis which followed show that liquid nitrogen-stored pollen of all three clones germinate as well as that of fresh pollen after one year of storage. It is therefore possible that although many, if not most, *Protea* pollen could be frozen in liquid nitrogen and thawed a number of times, it would be preferable to freeze a number of small samples rather than one large sample to ensure that the viability of an entire batch would not be affected. This should be checked for *Protea* pollen frozen at higher temperatures.

4.5 Literature cited

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Table 4.1 **Correlations between FCR percentage and germination percentage *in vitro* based upon the data sources used for the text figures listed**

Treatment		Source of data	r (N = 6)
Temperature	**Relative humidity**		
Room	0%	Fig. 4.7A & B	-0.42
22° to 27°C)	10%	Fig. 4.7A & B	-0.52
	30%	Fig. 4.7A & B	-0.55
	60%	Fig. 4.7A & B	-0.24
Fridge	0%	Fig. 4.7C & D	0.80
(2° to 7°C)	10%	Fig. 4.7C & D	0.05
	30%	Fig. 4.7C & D	-0.45
	60%	Fig. 4.7C & D	0.89*
Freezer	0%	Fig. 4.8A & B	-0.16
(-14° to -18°C)	10%	Fig. 4.8A & B	-0.62
	30%	Fig. 4.8A & B	-0.77
	60%	Fig. 4.8A & B	0.45
LN_2 (-196°C)	—	Fig. 4.8C & D	0.70

*P < 0.05

Fig. 4.1 A. Inflorescence of *P. repens* cv. 'Sneyd'. B. Inflorescences caged with semi rigid wire mesh to exclude pollinators. C. Close-up of caged inflorescence, showing ring of florets prepared for pollination.

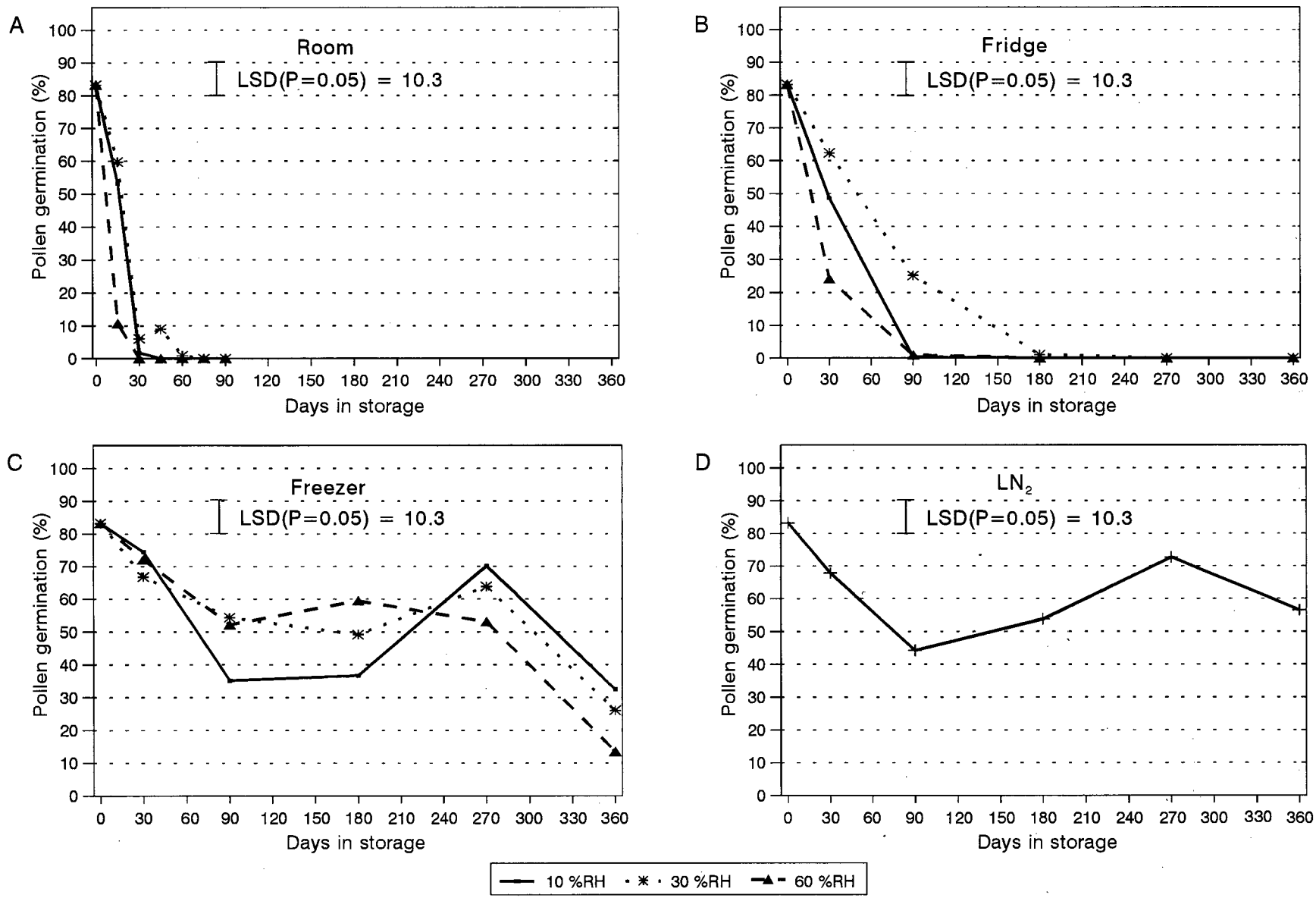

Fig. 4.2 The effect of storage at different temperatures and relative humidities on pollen germination of P. *aristata* cv. 'Aristocrat'. (A) Room temperature (22° to 27°C). (B) Fridge (2° to 7°C). (C) Freezer (-14° to -18°C). (D) Liquid nitrogen (-196°C).

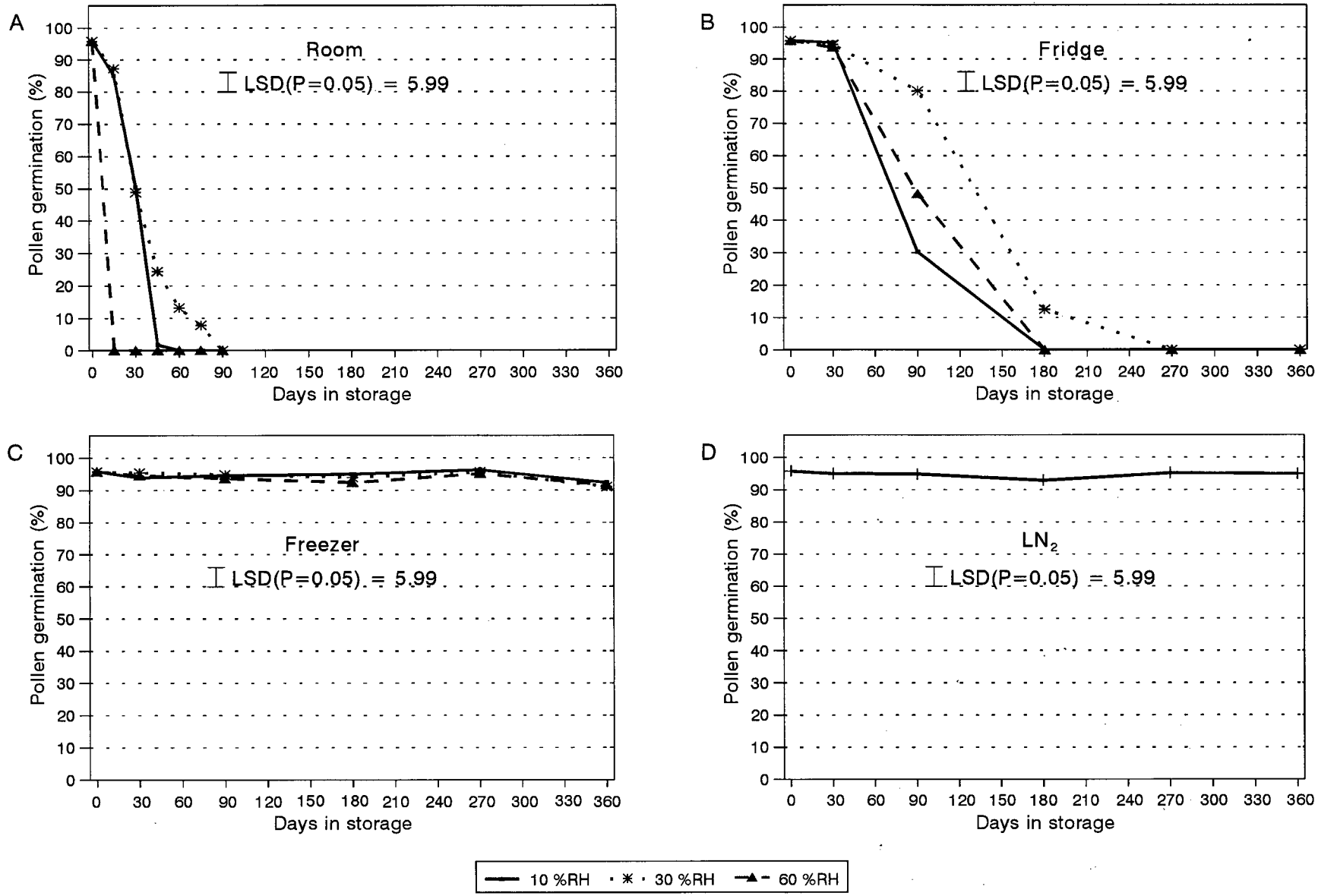

Fig. 4.3 The effect of storage at different temperatures and relative humidities on pollen germination of P. *eximia* cv. 'Fiery Duchess' (A) Room temperature (22° to 27°C). (B) Fridge (2° to 7°C). (C) Freezer (-14° to -18°C). (D) Liquid nitrogen (-196°C).

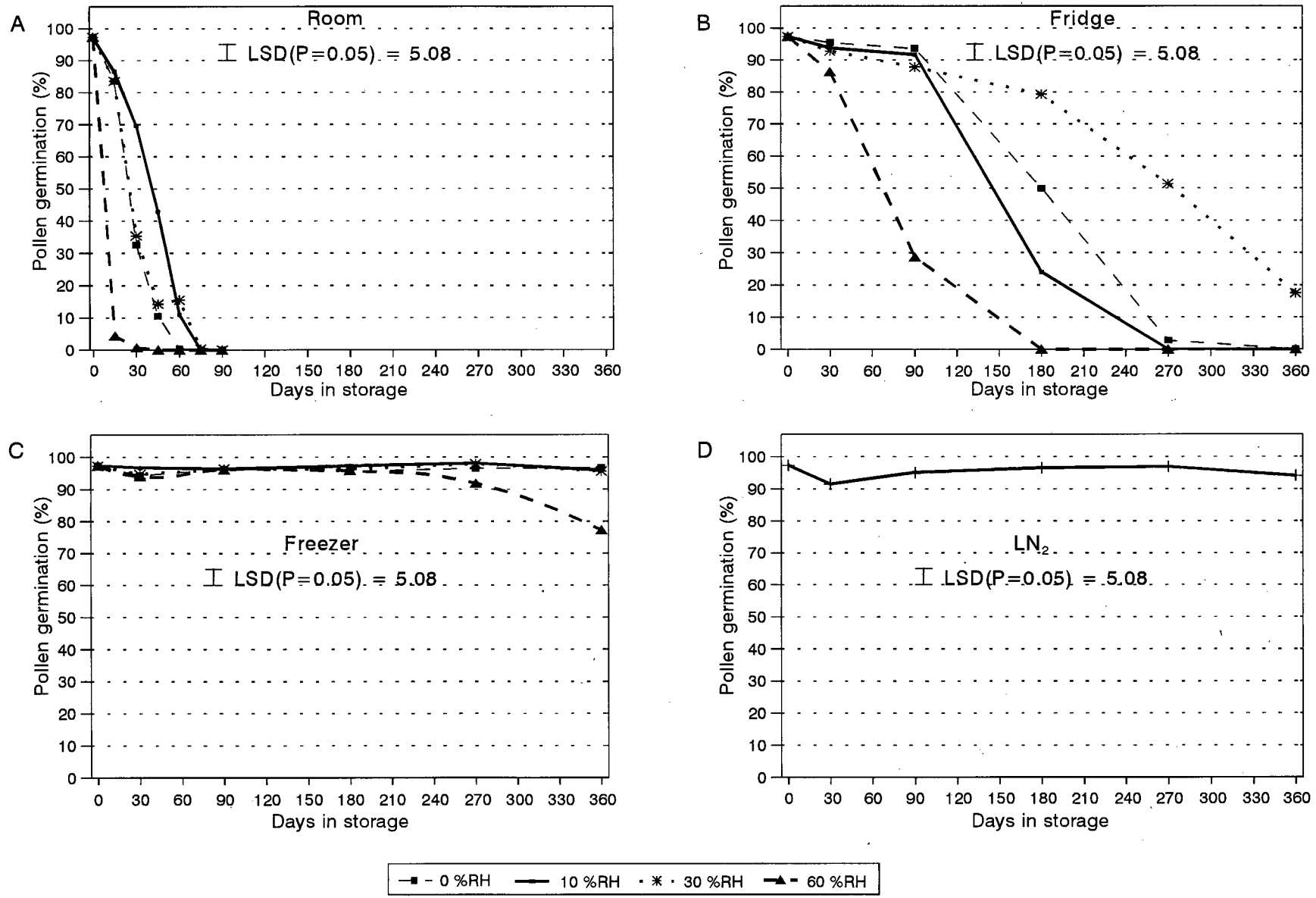

Fig. 4.4 The effect of storage at different temperatures and relative humidities on pollen germination of P. *repens* cv. 'Sneyd'. (A) Room temperature (22° to 27°C). (B) Fridge (2° to 7°C). (C) Freezer (-14° to -18°C). (D) Liquid nitrogen (-196°C).

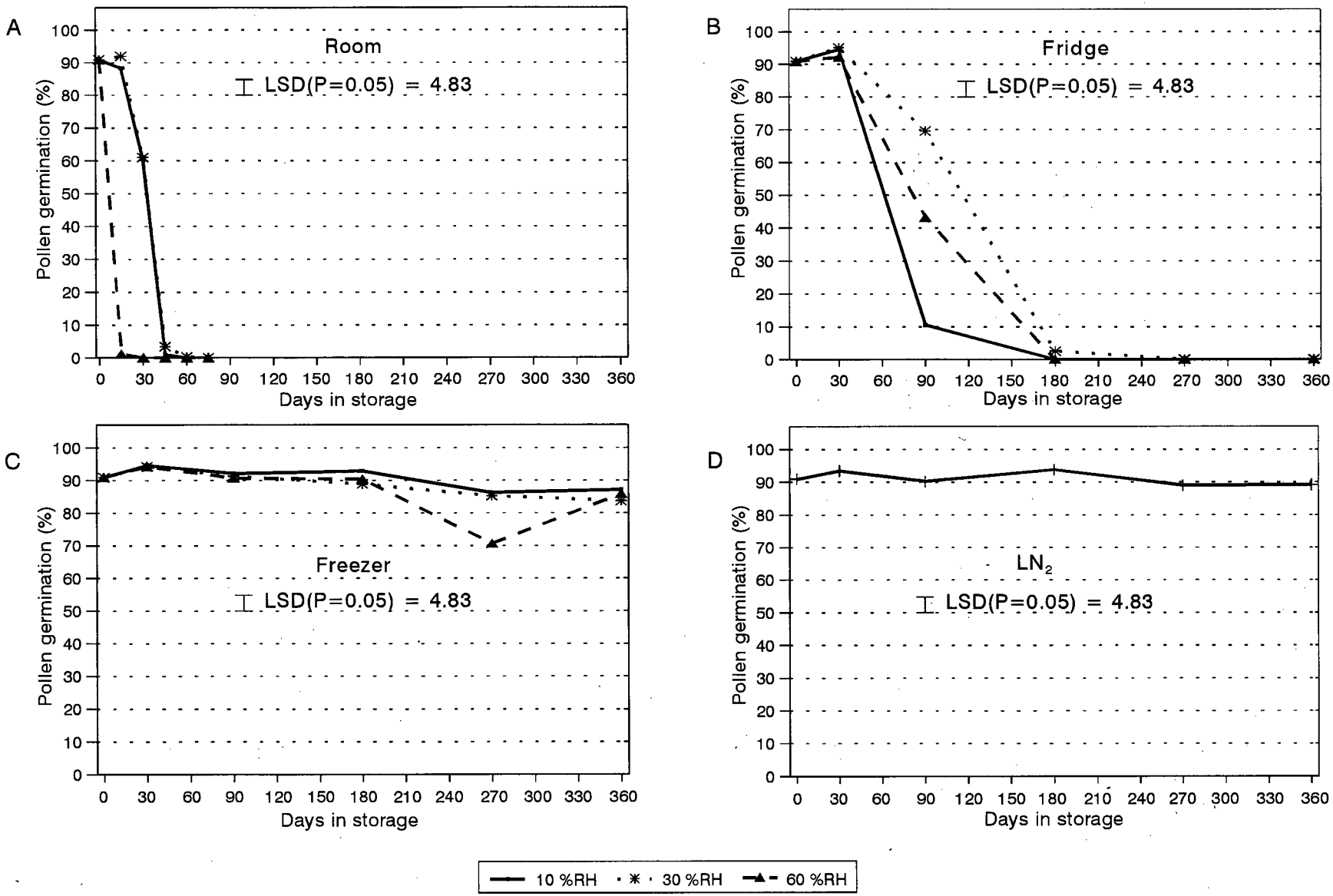

Fig. 4.5 The effect of storage at different temperatures and relative humidities on pollen germination of *P. magnifica* clone 'T840705'. (A) Room temperature (22° to 27°C). (B) Fridge (2° to 7°C). (C) Freezer (-14° to -18°C). (D) Liquid nitrogen (-196°C).

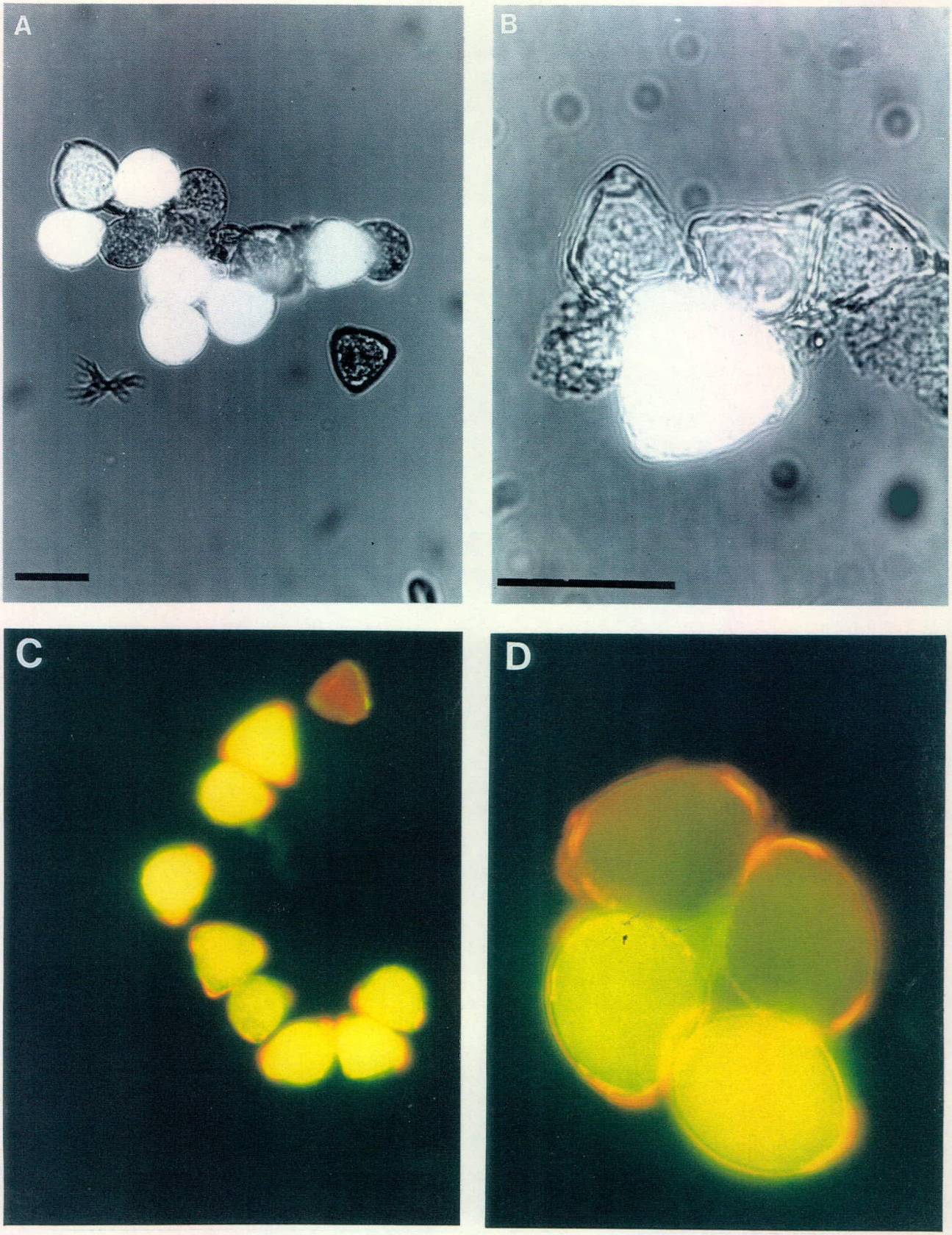

Fig. 4.6 Fluorescence micrographs of stored *P. repens* cv. 'Sneyd' pollen, stained with Fluorescein-diacetate/Propidium-iodide. A. Micrograph showing six viable (fluorescing) pollen grains and seven dead grains. x 400. B. Micrograph showing one viable (fluorescing) grain and three dead grains. x 1000. C. Colour micrograph showing the bright yellow fluorescence of viable pollen grains and the reddish-brown colour of dead pollen. x 400. D. Colour micrograph showing two viable and two dead grains. x 1000. Bars = 30 μm.

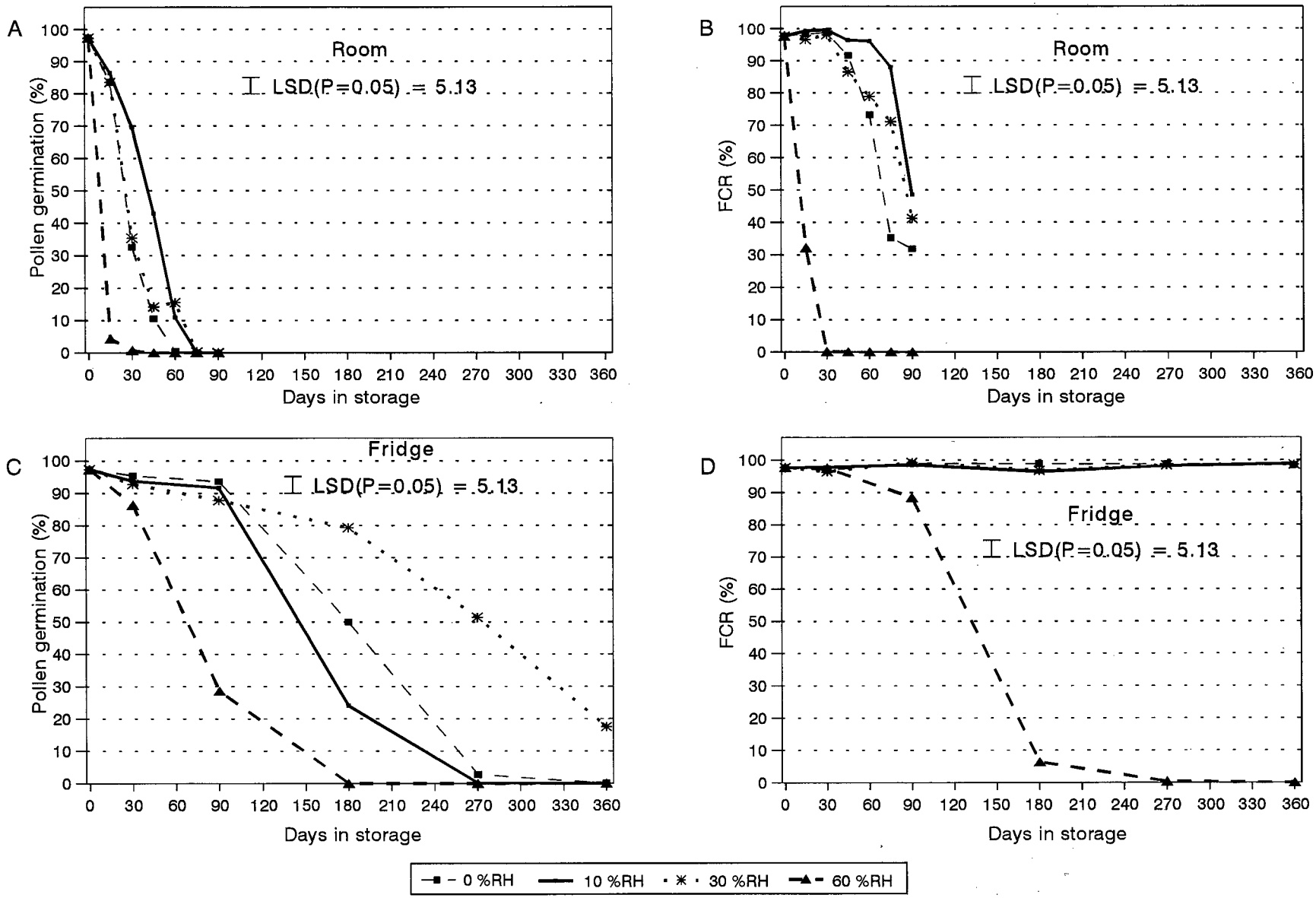

Fig. 4.7 Comparison of germinability and fluorochromatic reaction (FCR) in pollen samples of *P. repens* cv. 'Sneyd' stored at different temperatures and relative humidities. (A) Germination after room temperature storage (22° to 27°C). (B) FCR after room temperature storage (22° to 27°C). (C) Germination after fridge storage (2° to 7°C). (D) FCR after fridge storage (2° to 7°C).

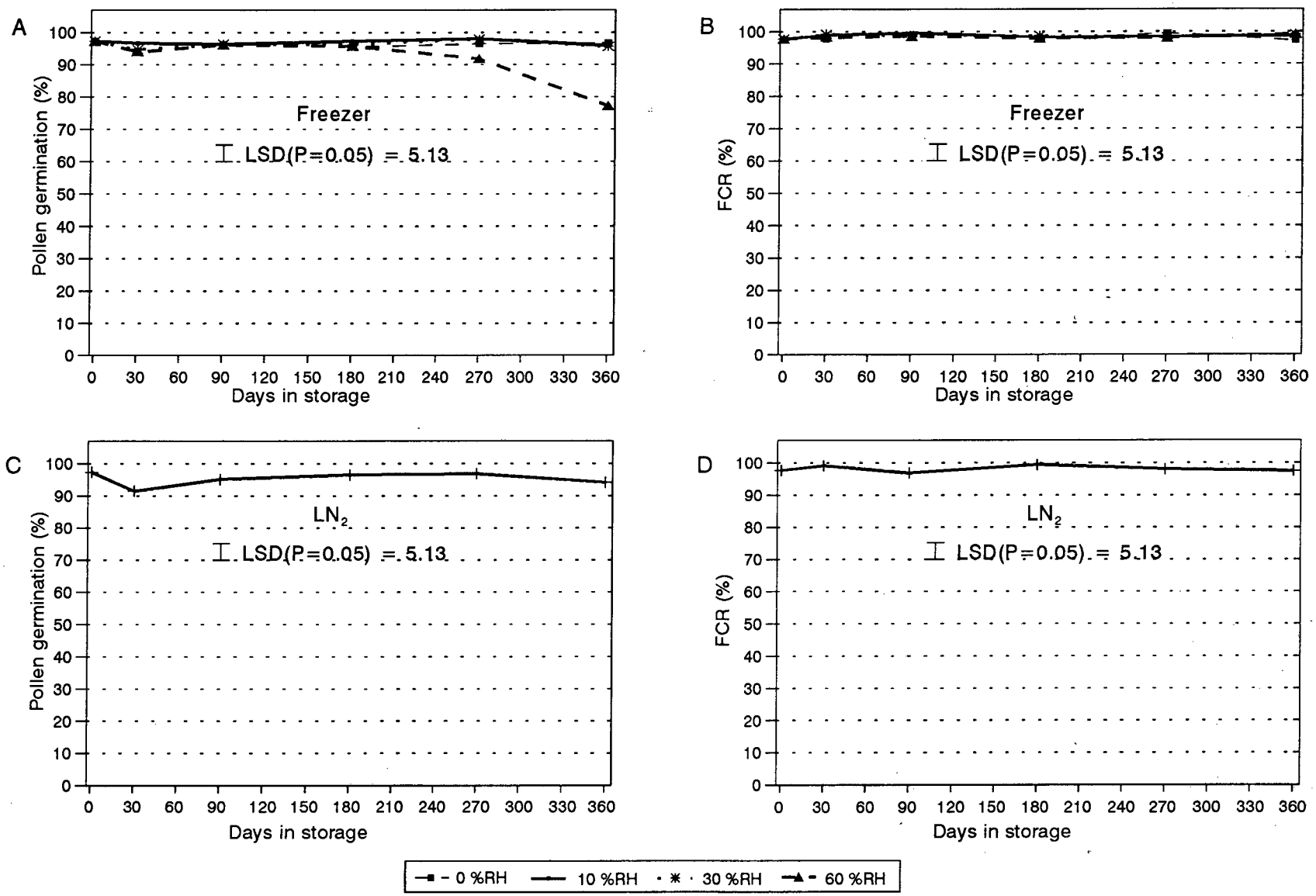

Fig. 4.8 Comparison of germinability and fluorochromatic reaction (FCR) in pollen samples of *P. repens* cv. 'Sneyd' stored at different temperatures and relative humidities. (A) Germination after freezer storage (-14° to -18°C). (B) FCR after freezer storage (-14° to -18°C). (C) Germination after liquid nitrogen storage (-196°C). (D) FCR after liquid nitrogen storage (-196°C).

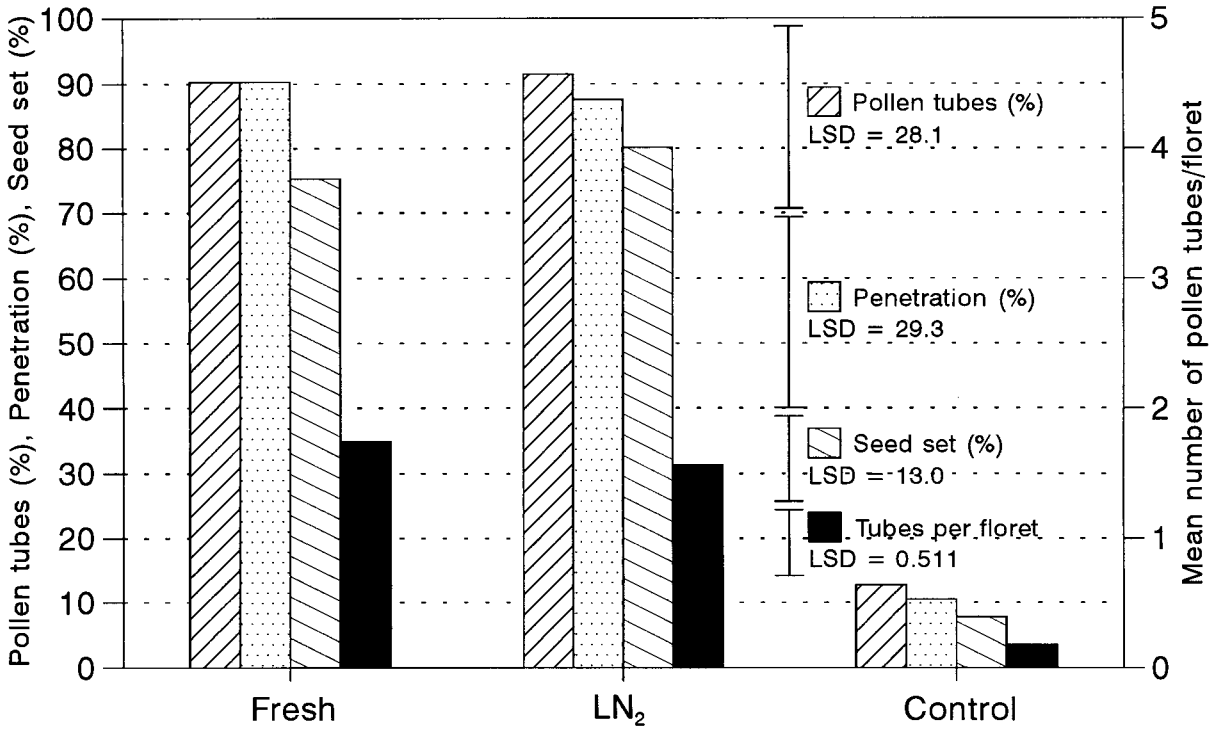

Fig. 4.9 Fertilization success of fresh and 15 month old liquid nitrogen-stored *P. repens* cv. 'Sneyd' pollen after hand pollination, measured by: (1)percentage of florets with pollen tubes, (2)percentage of ovules penetrated by a pollen tube, (3)percentage seed set and (4)mean number of pollen tubes per floret.

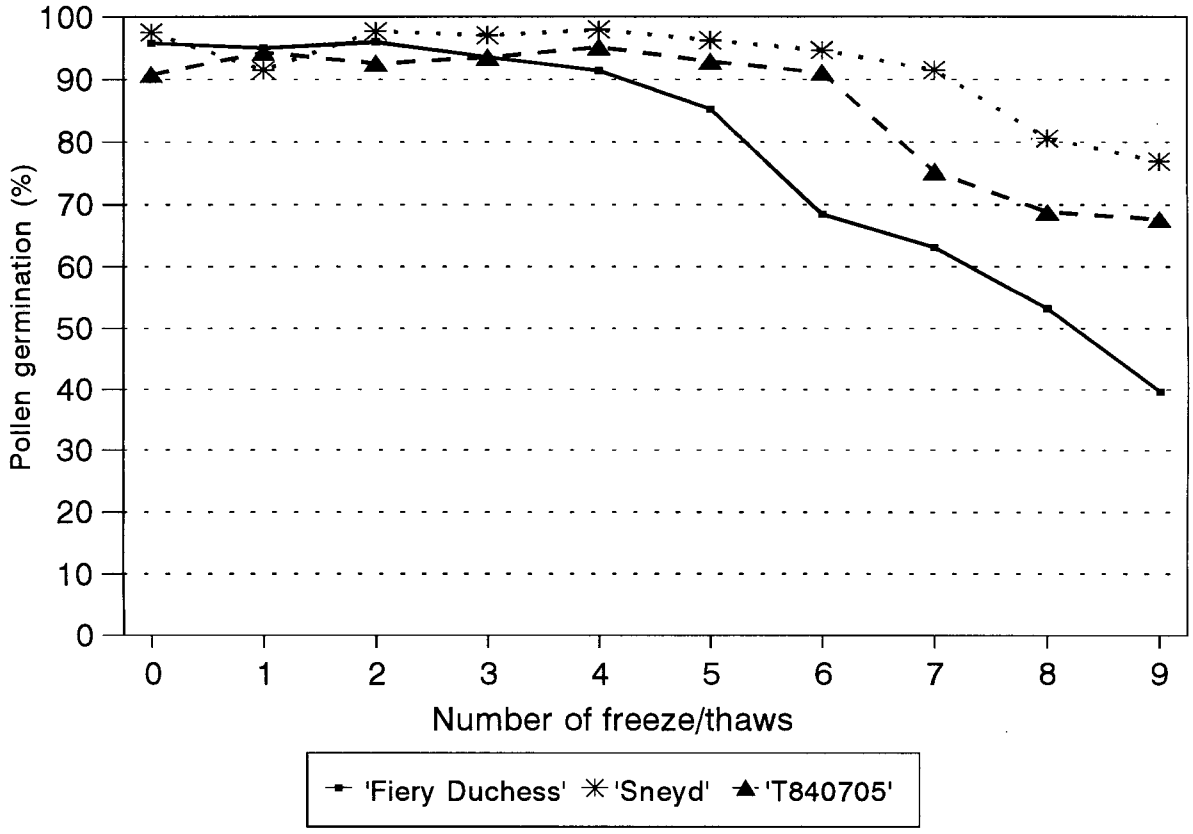

Fig. 4.10 Effect of repeated freeze/thawing on germinability of *P. eximia* cv. 'Fiery Duchess', *P. repens* cv. 'Sneyd' and *P. magnifica* clone 'T840705' pollen.

CHAPTER 5

POLLEN MORPHOLOGY, MALE HYBRID FERTILITY, POLLEN TUBE PATHWAYS AND PRELIMINARY INVESTIGATIONS ON BREEDING SYSTEMS INCLUDING INTERSPECIFIC CROSSING IN *PROTEA*

Abstract: The morphology and size of *Protea* pollen was studied, using light and scanning electron microscopy. There was a high level of consistency in pollen grain exomorphology between different species and clones. Polymorphic grains were observed in two interspecific hybrids. Negligible differences in pollen grain size were recorded between clones and species. The male fertility of 25 interspecific *Protea* hybrids, based on pollen germinability *in vitro*, was investigated. The majority of hybrids were found to be sufficiently fertile for use in a breeding programme. However, hybrids with *P. cynaroides* were totally sterile. Pistil structure and pollen tube pathways were investigated in *P. repens* cv. 'Sneyd' using light and scanning electron microscopy. The pistil has four distinct regions consisting of the stigma, the club-shaped upper style, the heart-shaped lower style, and the ovary. The distal part of the pistil is modified to form the pollen presenter area with a longitudinal, obliquely terminal groove down one side. The groove contains the stigma papillae cells. The pistil has a stylar canal along its entire length and this canal is also the route by which pollen tubes grow to the ovary. The upper style is heavily lignified with sclerenchyma tissue which makes pollen tube observations very difficult. Very low numbers of pollen tubes actually reach the ovary. Natural breeding systems of *P. repens* cv. 'Sneyd' and *P. eximia* cv. 'Fiery Duchess' were deduced from pollen tube and seed set data after controlled hand-pollinations. Both clones were found to be fully self-compatible. Very low percentages of autogamous seed set were recorded. Interspecific crosses were unsuccessful. It is suggested that the incompatibility reaction occurrs on the stigma and/or in the upper style region.

5.1 Introduction

Studies on natural systems of breeding such as autogamous propagation, self-pollination and cross-pollination (including interspecies hybridization) are fundamental to the formulation of systematic controlled plant breeding programmes, and often involve studies on the growth of pollen tubes in stylar tissue (Pandey and Henry, 1958) an on numbers of seed set. The fertility of interspecies hybrids is of particular interest in *Protea* and has thus far not been studied.

Studies on pollen growth in pistils are necessary to understand the causes of poor seed set. In many species, pollen tubes can easily be stained and some standard techniques have been developed (e.g., Martin, 1959; Kho and Baër, 1968). Pistil structure and pollen have been described for *Protea cynaroides* (Vogts, 1971). Detailed descriptions of the pistil structure and pollen tube growth have also been described for many species of genera of the Australian Proteaceae, e.g., *Banksia, Grevillea* and *Macadamia* (Sedgley *et al.*, 1985; Herscovitch and Martin, 1989; Fuss and Sedgley, 1991b; Clifford and Sedgley, 1993; Sedgley *et al.*, 1993). Both the presence of pollen tubes in pistils and the formation of seeds have been used in breeding system studies in order to determine the success of autogamy and/or self-pollination treatments (Lewis and Bell, 1981; Collins and Spice, 1986; Vaughton, 1988; Goldingay and Whelan, 1990).

The objectives of this study were, (1) to describe the exomorphology of *Protea* pollen, (2) to investigate male fertility of interspecific hybrids, (3) to develop a method of measuring pollen tube growth in the pistil, and (4) to investigate alternative breeding systems including interspecific crosses. The possibility that interspecific crossing may be impaired by size differences of pollen of different species was also investigated.

5.2 Material and Methods

5.2.1 *Plant material*

All the experiments were conducted during the 1993 and 1994 flowering seasons on two mature (7-year-old) bearing *Protea* clones planted in experimental plantations at Elsenburg

(latitude 33°51'S, longitude 18°50'E; 177 m a.s.l.) and Riviersonderend (latitude 34°08'S, longitude 19°54'E; 168 m a.s.l.) in South Africa. Prior to the experimental period all plants had been subjected to routine plantation management practices, including drip-irrigation during the summer months. Harvesting of blooms in previous years served as the only form of pruning of the bushes.

5.2.2 *Pollen morphology and grain size*

For exomorphology studies of *Protea* pollen, pollen of *P. repens* cv. 'Sneyd', *P. eximia* cv. 'Fiery Duchess', *P. neriifolia* cv. 'Red Robe', and an interspecific hybrid, *P. obtusifolia* x *P. compacta* cv. 'Red Baron', were examined. Fresh pollen samples were collected from the four cultivars and dispersed in 100% ethanol solutions. Drops of these suspensions were placed on aluminium stubs, left to evaporate, sputter-coated with gold, and examined with a Jeol JSM6100 scanning electron microscope (SEM) operated at 5 kV.

For the determination of the relative sizes of pollen of different *Protea* species and hybrids, fresh pollen samples of 21 clones were collected. Seven inflorescences from each of seven different plants of the same clone were harvested when approximately one half of the florets have undergone anthesis. The inflorescences were brought to the laboratory where the stems were placed in water and all open florets were removed with scissors. Sixteen hours later all florets that had subsequently opened were harvested (c. 25 per inflorescence). The pollen of each inflorescence was scraped off and thoroughly mixed to give a uniform pollen sample. Before measuring, pollen samples were placed in a 100% relative humidity chamber for three hours to rehydrate. After rehydration, pollen samples were mounted in glycerine jelly according to the procedure of Moore *et al.* (1991). Pollen were measured under oil immersion at a magnification of x 1000 with an Olympus BH-2 light microscope equipped with a calibrated eyepiece. The first 20 randomly selected grains with the correct orientation (polar view) were measured. The length of the polar axis was taken as an index of pollen size.

5.2.3 *Hybrid pollen fertility*

The male fertility of 25 interspecific (F_1) *Protea* hybrids, based on their *in vitro* germinability, was investigated under a light microscope by means of the hanging-drop technique. The medium used for the germination of the pollen was the same as that used for pollen storage experiments (Chapter 4). The collection, sampling, and rehydration of pollen were caried out in the same manner as described above, except that in this case five plants per clone were used. Germination and sterile grains (empty shells) for each clone were scored after three hours by means of an Olympus BH-2 light microscope. A minimum of 200 randomly selected pollen grains in four different fields was scored for germination and only pollen grains producing tubes longer than the grain diameter were scored as germinated.

5.2.4 *Pistil structure and pollen tube pathways*

Pollen tube growth was examined in *P. repens* cv. 'Sneyd', using cross-pollinated flowers.

5.2.4.1 Light microscopy

Pistils for paraffin sectioning were harvested seven days after pollination. They were cut into 1 cm pieces and ovaries were carefully dissected out of the involucral receptacle. Material was fixed in Carnoy's solution for at least 24 hours (Sedgley *et al.*, 1985), dehydrated in an ethanol series, embedded in paraffin wax (Anglia Scientific, cat no. 1551), and sectioned (5 – 10 μm) with an Anglia Scientific 500 rotary microtome. Both longitudinal and transverse sections were cut. Aniline blue-UV induced fluorescence was used to identify callose associated with the pollen tube wall (Smith and McCully, 1978). Paraffin sections were deparaffinized in xylene for two hours, washed three times in distilled water, stained with 0.1% aniline blue in 0.1N $K_3PO_4.H_2O$ buffer for two hours, sealed with 80% glycerin, and examined with a Nikon Biophot microscope equipped with episcopic-fluorescence as described previously (Chapter 4). Fluorescence micrographs were produced using Agfa XRG ASA 400 colour film. Some sections were also mounted on slides and stained for general observation with alcian green-safranin (Joel, 1983). Slides were examined with a Vanox AH-

2 Olympus Photomicroscope, and photomicrographs were produced using Fuji ASA 100 colour film.

Pollen presenters for embedding in Spurr's low viscosity medium (Spurr, 1969), were harvested seven days after pollination, fixed in 2.5% glutaraldehyde containing 0.5% caffeine in 0.07 M phosphate buffer, pH 7.2, for 2 hours (Glauert, 1975; Mueller and Greenwood, 1978; Hayat, 1986). Following postfixation in 0.5% osmium tetroxide (OsO_4) for one hour, rinsing in two changes of double distilled water, the tissue was dehydrated in an acetone series and then embedded in Spurr's resin. Tissue was sectioned at 2 – 3 μm with a diamond knife on a Sorvall MT 5000 Ultramicrotome, and selected sections were mounted on slides and stained with Toluidine Blue O (0.5% in acetate buffer, pH 4.5) (Gabriel, 1982). Slides were examined with a Vanox AH2 Olympus Photomicroscope and photomicrographs were produced using Fuji ASA 100 colour film.

5.2.4.2 Scanning electron microscopy (SEM)

Pollen presenters for scanning electron microscopy were harvested two days after pollination, mounted on aluminium stubs, using double-sticking tape and silver paste, frozen in nitrogen slush, and sputter-coated with gold in an Oxford CT 1500 Cryo-trans-system before transfer to the cold stage of the Jeol JSM6100 SEM operated at 4 kV. Photomicrographs were produced using Ilford FP4 black and white film.

5.2.5 *Breeding system*

Preliminary investigations on breeding systems were carried out in two separate experiments involving *P. repens* cv. 'Sneyd' and *P. eximia* cv. 'Fiery Duchess' respectively by excluding pollinators from flowering inflorescences and by hand-pollinating inflorescences. Both experiments involved preparing, caging, and pollinating 50 inflorescences (10 replicates) three days after anthesis as described previously (Chapter 4). The pollination treatments in the experiment with cv. 'Sneyd' were as follows:

1) Autogamy (control); inflorescences were caged and left unpollinated.

2) Self-pollination; pollen from flowers of the same clone (cv. 'Sneyd') was applied to the stigma.

3) Intraspecific cross-pollination; pollen from another *P. repens* clone (T 89 05 02) was applied to the stigma.

4) Interspecific cross-pollination; pollen from another *Protea* species (*P. eximia* 'T 76 03 06') was applied to the stigma.

5) Interspecific cross-pollination; pollen from a *Protea* F_1 hybrid (*P. compacta* x *P. susannae* cv. 'Pink Ice') was applied to the stigma.

Three replicates (15 inflorescences) were harvested seven days after pollination when penetration of the ovule had occurred. The ovary part of the pistil was carefully dissected out of the involucral receptacle and fixed in Carnoy's solution. Ovaries were prepared for fluorescence microscopy as described previously.

The effectiveness of each pollination treatment was assessed by counting the number of tubes reaching the ovary, calculating the percentage ovaries containing pollen tubes, and calculating the percentage of ovules which had been penetrated.

The rest of the replicates (35 inflorescences) were left on the plants for seven months until the achenes had matured (Van Staden, 1978). The percentage seed set of pollinated florets per seed-head was determined as described previously.

In the experiment with cv. 'Fiery Duchess', the pollination treatments were as follows:

1) Autogamy (control): inflorescences were caged and left unpollinated.

2) Self-pollination: pollen from flowers of the same clone (cv. 'Fiery Duchess') was applied to the stigma.

3) Intraspecific cross-pollination: pollen from another *P. eximia* clone ('T 85 02 03') was applied to the sigma.

4) Interspecific cross-pollination: pollen from another *Protea* species (*P. cynaroides*) was applied to the stigma.

5) Interspecific cross-pollination: pollen from a *Protea* F_1 hybrid (*P. eximia* x *P. susannae* cv. 'Cardinal') was applied to the stigma.

No pollen tube observations were made in this experiment and all 45 inflorescences were left on the plants for seed set determinations.

5.2.6 *Experimental design and statistical analysis*

The pollen grain size experiment consisted of 21 treatments (clones) in seven randomized blocks. The hybrid pollen fertility experiment consisted of 25 treatments (clones) in five randomized blocks. The breeding system experiment with cv. 'Sneyd' consisted of five treatments in 10 randomized blocks. The experiment was conducted in two parts and analysis of variance (ANOVA) was performed for each part of the experiment separately. Pearson product-moment correlations were calculated to measure the strength of the linear relationship between different variables connected to pollen tube-growth. The breeding system experiment with cv. 'Fiery Duchess' consisted of five treatments in nine randomized blocks.

Analysis of variance for all the experiments, was performed using SAS statistical software version 6.08 (SAS Institute Inc., Cary, NC, USA). Student's least significant difference (LSD) was calculated at the 5% probability level to compare treatment means. For all other effects in the analysis of variance a probability level of 5% was considered significant.

5.3 Results

5.3.1 *Pollen morphology and grain size*

Mature *Protea* spp. pollen grains are triporate and subisopolar with one polar face more rounded convex, and apertures displaced towards the less convex surface (Fig.5.1A; 5.1B). The grains are triangular in polar view, with the amp straight and obtuse (Fig. 5.1A). The three apertures are circular, relatively small (c. 7 μm), non-bordered and located at each corner of the triangular grain. The exine is rough and pitted (tectate-foveolate). Spherical wart-like sculpturing elements (orbicules ≡ Ubisch bodies), about 1 μm high, are scattered at irregular distances (often in clumps) on the exine (Fig. 5.1). The exine encircling the pores are psilate and imperforate.

There is a high level of consistency in pollen grain structure in the species. However, in two of the interspecific hybrids examined, polymorphic grains have been observed. Both cv. 'Red Baron' and cv. 'Patrysie' have three-porate (triangular, c. 25%), four-porate (quadrangular, c. 60%) (Fig.5.1E), and five-porate (quinquangular, c. 15%) grains (Fig. 5.1C; 5.1D). The rest of the exo-morphological features are the same as the normal three-porate grains except that the amp is concave instead of straight (Fig. 5.1C; 5.1E). These aberrant-type grains are fully fertile and pollen tubes often grew out of these grains from two apertures simultaneously, in contrast to the tube growth of triangular grains which is almost always restricted to one pore.

Results of the pollen grain diameter measurements of different clones/species are given in Table 5.1. Analysis of variance showed significant clone differences ($P < 0.01$) in pollen grain diameter. The maximum and minimum diameters recorded were 35.3 μm (*P. neriifolia*, clone 'T 73 05 02') and 26.8 μm (*P. pudens* x *P. burchellii*, clone 'T 94 06 05') respectively. Small but significant differences were found between most clones of the same species. Moreover, hybrids did not have bigger pollen grains than the parental species.

5.3.2 *Hybrid pollen fertility*

Results of the male fertility of interspecific *Protea* hybrids, based on pollen germinability *in vitro*, are shown in Table 5.2. Analysis of variance showed significant differences between hybrid clones ($P < 0.01$) in the germinability score. Germinability ranged from > 80% to zero. Eleven of the 25 hybrids tested had a germinability exceeding 60%. There appears to be no connection between the species of a cross and the germinability of the pollen, except in the case of the two hybrids involving *P. cynaroides* as one parent. The pollen of both of these hybrids was completely sterile. Most hybrids tested had a high percentage sterile (empty shells) pollen grains. These grains had a distinct morphology and could easily be distinguished from normal fertile pollen grains (Fig. 5.2A). The correlation between percentage germination and percentage sterile grains was negative ($r = -0.84$) and highly significant ($P < 0.01$). Hybrids with high pollen germinability had relatively few sterile grains, while those with low germinability had larger numbers of sterile grains. However, some hybrids had a low to moderate germinability despite having a low percentage of sterile grains (Table 5.2).

5.3.3 *Pistil structure and pollen tube pathways*

5.3.3.1 Pistil structure

The cv. 'Sneyd' inflorescence contained an average of 110 ± 3.42 florets, and each pistil was 80 ± 3.81 mm long. The pistil was found to have four major regions, consisting of the stigma, a vertebra-shaped upper style, a heart-shaped lower style, and the ovary.

The upper part of the pistil appears to be modified to form the pollen presenter area consisting out of an elongated ridged structure, where pollen is deposited prior to anthesis, and a longitudinal, obliquely-placed terminal groove (c. 420 μm in length) on the upper adaxial side of the pistil (Fig. 5.2C). The margins of the groove appear serrated and fringed by a layer of stigma papillae cells which interlock with each other (Fig. 5.2B). The stigma was dry and no secretion of stigmatic exudate was observed at anthesis.

A transverse section through the vertebra-shaped upper style, stained with alcian green-safranin, is illustrated in Figure 5.2D. The distinctive vertebra-shape appears to be due to the elongated ridge structure with the troughs corresponding to the location of anther lobes in the unopened flower. The tissues of the upper style from the outer surface, consist of the epidermis, polyphenol-containing cells, a thick layer of sclerenchyma cells, large parenchyma cells, and one vascular bundle. An oval-shaped stylar canal is located adaxially to the vascular bundle near the sclerenchyma layer, connected to the stigmatic groove. The stylar canal appears to be surrounded by small, densely-packed outer transmitting tissue (ott) cells (Fig. 5.2E; 5.4C; 5.4D). The stylar canal appears to consist of two parallel rows of approximately 10 large (5 — 8 μm diameter) thin-walled inner transmitting tissue (itt) cells aligned with the groove in the pistil (Fig. 5.2E; 5.4C). A small gap of approximately 10 μm is maintained between the two files of itt cells (Fig. 5.2E; 5.4C; 5.4D). The interlocking structure observed in the papillae cells was also evident in the inner tt cells, and their structures appear very similar.

A transverse section through the style, at a point half-way down, stained with alcian green-safranin, is illustrated in Figure 5.3A. At this point, the lower style has a diameter of approximately 1 040 μm and contains nine vascular bundles with the major bundle abaxial to the stylar canal. The lower style has many more sclerenchyma cells (c. five layers), making the tissue woody and causing difficulties in sectioning this part of the style. The stylar canal with its tt cells appears to persist throughout the whole length of the pistil (Fig. 5.3). Towards the base of the style the sclerenchyma cells disappear, the style becomes less woody and sectioning is easier. The ring of polyphenol cells also disappears and only a small number of these cells are randomly distributed throughout the cortex (Fig. 5.3C). The stylar canal joins up with the cavity formed between the ovule and the inner ovary wall (Fig. 5.3C; 5.4B).

Longitudinal and transverse sections through the ovary are shown in Figures 5.3D and 5.4A respectively. The ovary was partially embedded in the woody involucral receptacle of the inflorescence (Fig. 5.4A), and appears to contain one acutely obovate-shaped ovule.

5.3.3.2 Pollen tube pathways

The stigmatic groove was observed to be always narrower than the pollen grain diameter. Germination was only observed in pollen grains located in or near the groove (Fig. 5.5B; 5.5C; 5.5D) and pollen grains were often seen to be wedged sideways into the groove (Fig. 5.5A). However, despite an abundance of pollen grains near the groove, very few germinated and very few pollen tubes were observed growing into the stigmatic groove (Fig. 5.5B; 5.5C; 5.5D).

The pollen tubes grew in close proximity to each other between the two files of inner tt cells of the stylar canal (Fig. 5.6A; 5.6B). The woody nature of the pistil and the extremely bright autofluorescence of the vascular bundles (Fig. 5.6E; 5.7F) made it very difficult to locate pollen tubes in the upper part of the pistil. Therefore, most pollen tube observations (squashes) were done with the relative soft ovary part of the pistil. Pollen tube growth appears to be confined to the stylar canal for the whole length of the style. Qualitative observations indicate that the first pollen tubes reach the ovary four days after pollination, with maximum pollen tube numbers recorded seven days after pollination, implying that the fastest tubes grew at a rate of approximately 0.83 mm/h. On average, one to four pollen tubes reached the space between the inner ovary wall and the ovule (Fig. 5.6C; 5.6D). It appears that the tubes then grow along the surface of the inner ovary wall (Fig. 5.7A; 5.7B) down to the micropyle where one pollen tube alone makes a 180° turn beneath the micropyle (Fig. 5.7C; 5.7D). The pollen tube penetrates the ovule by growing between the papillate cells of the apex of the nucellus (Fig. 5.7A; 5.7E).

No abnormalities were observed in pollen tubes reaching the ovary in any of the crosses of this study. Pollen tubes were straight and smooth-walled. They did not produce callose plugs and fluoresced brightly along their entire length, indicating that callose is uniformily distributed along the pollen tube wall (Fig. 5.6E; 5.6F).

5.3.4 *Breeding system*

Results of the experiment with cv. 'Sneyd' are illustrated in Figure 5.8. Analysis of variance showed a significant treatment effect ($P < 0.01$) for all three pollen tube variables tested as well as for seed set percentages. There were no significant differences between the effects of self-pollination and cross-pollination (intraspecific), nor between the control (autogamy) and the interspecific cross-pollinations, but there were significant differences between the two pairs of treatments for all four variables. Correlation coefficients for the three pollen tube variables were all highly significant ($P < 0.01$).

Very few pollen tubes actually reach the ovary part of the pistil. On average, less than 0.5 pollen tubes were observed to have reached the ovary in the control and interspecific pollinations, compared to 2.7 in the intraspecific pollinations. Very high percentages of ovaries containing pollen tubes ($> 81\%$) and ovaries with penetrated ovules ($> 65\%$) were observed in both the self- and intraspecific cross-pollination treatments. Seed set percentages were not significantly different from the penetration percentages obtained. Seed set was very high ($> 65\%$) for the self- and intraspecific pollinations compared to low seed set ($< 18\%$) in the other pollinations.

Results of the seed set experiment with cv. 'Fiery Duchess' are presented in Table 5.3 for which analysis of variance shows a significant treatment effect ($P < 0.01$). Seed set percentages over treatments were found to be extremely low ($< 11\%$) (see also Chapter 6). and no significant differences were found between the effects of self-pollination, intraspecific cross-pollination and cross-pollination with the *P. eximia* hybrid, nor between the control (autogamy) and interspecific cross-pollination. However, the observed differences between the two pairs of treatments are significant. Only two inflorescences produced seed in the interspecific cross-pollination (1.3%), while no seeds were produced in the control (autogamy).

5.4 Discussion

This study has shown that there is very little variation in *Protea* spp. pollen structure and size. The triangular grain is most common and is seen in 55 genera of nine tribes of the Proteaceae family (Venkata Rao, 1971). *Leucadendron argenteum* has the largest mature pollen grain (45 – 61 μm diameter) of the South African Proteaceae (Garside, 1946). The polymorphic grains observed in the two interspecific hybrids form an aberrant type of uncertain origen. However, polymorphism in pollen structure has previously been observed in *Grevillea vestita* (Proteaceae) by Venkata Rao (1971).

The surface area of the *Protea* stigmatic groove is very small (Vogts, 1971; Rebelo and Rourke, 1986). In *Banksia*, for germination to occur, the pollen grains have to be located in the groove itself (Fuss and Sedgley, 1991a). Vogts (1980; 1982) postulated that *Protea* pollen had to be forced into the groove by pollen vectors to effect pollination. This study found very small differences in pollen grain sizes between species and indicates that pollen germination can occur on the outside of the groove. By implication it then seems unlikely that differences in pollen grain size prevent interspecific pollen from being deposited into the stigmatic groove and therefore contribute to the poor results obtained in artificial interspecific hybridizations with *Protea* in the past (Brits, 1983; Brits and Van den Berg, 1990).

This study has also shown that the pollen of most interspecific hybrids is sufficiently fertile for use in breeding programmes. *Protea cynaroides* is considered to be the most primitive species in the genus *Protea*, and is classified on its own in the infrageneric classification of *Protea* (Rourke, pers. comm.). This large infrageneric distance between *P. cynaroides* and the other species may explain the sterility of the two *P. cynaroides* hybrids found and should be studied further in other crosses.

The pistil structures of *Protea repens* are very similar to those described in *Protea cynaroides* (Vogts, 1971), *Macadamia* (Sedgley *et al.*, 1985) and *Banksia* (Clifford and Sedgley, 1993). However, in contrast to the pistils of *Macadamia* and *Banksia* which have only a partial stylar canal, the *Protea repens* pistil has a definite stylar canal along its entire

length and this canal is also the route by which pollen tubes grow towards the ovary. Difficulties in observing pollen tubes were encountered because the stylar tissue is heavily lignified with sclerenchyma tissue. No techniques described in the literature to overcome the toughness of the style (e.g. incubating with pectinase or bisecting the style longitudinally) have been found satisfactory (Pandey and Henry, 1958; Fuss and Sedgley, 1991b). In this study the ovary was the only part of the *Protea* pistil where pollen tubes could clearly be observed. As in other members of the Proteaceae (Herscovitch and Martin, 1989; Fuss and Sedgley, 1991a; b), pollen tube numbers in the style were found to be extremely low, with rarely more than three pollen tubes per style. All the *Protea* ovaries examined contained a normal ovule, and this adds further support to the conclusion by Walker and Whelan (1991) and Clifford and Sedgley (1993) that andromonoecy is not a major cause of low seed set in Proteaceae.

This study shows that the two *Protea* species examined are fully self-compatible, in contrast to the findings of Horn (1962) who found self-incompatibility in *Protea* spp. The reason for this discrepancy is not clear and other *Protea* species should be studied further with respect to self-compatibility in the genus. The family Proteaceae shows variable levels of self-compatibility, viz. *Grevillea barklyana* (Ayre *et al.*, 1994), some species of *Leucospermum* (Horn, 1962; Rourke, 1972; Lamont *et al.*, 1985; Brits and Van den Berg, 1990), and *Serruria florida* (Horn, 1962) are self-compatible. On the other hand, a number of *Leucospermum* species (Horn, 1962; Lamont *et al.*, 1985; Brits and Van den Berg, 1990), and *Banksia* species (Collins and Spice, 1986; Vaughton, 1988; Ramsey and Vaughton, 1991) are self-incompatible. The extremely low autogamous seed set obtained in this study indicates that cross-breeding can be carried out without previous emasculation. Moreover, self-pollination breeding methods can be employed.

As is the case of the present study, disappointing results have thus far been obtained with artificial interspecific hybridization in the genus *Protea* (Brits, 1983). Chromosome numbers within proteaceous genera are constant (De Vos, 1943) and it appears from pollen tube and seed set data that hybridization is minimized by incompatibility reactions before fertilization. The actual site of incompatibility could not be determined in this study because pollen tube growth could not be followed in the upper style. However, it seems likely that the upper

style or stigma may be the site of pollen tube inhibition in interspecific *Protea* crosses, because no pollen tube abnormalities have been observed in the ovary, but this should be investigated further. Seed set percentages in all treatments in this study differed only slightly from the percentage of ovules penetrated by pollen tubes, indicating an absence of post-fertilization problems in *Protea* spp. The high percentage seed set obtained in the self and intraspecific crosses in cv.'Sneyd' also indicate that the pollination technique is not the cause of low seed set in observed interspecific crosses.

A prerequisite for future studies should be development of a method for observing pollen tubes in the upper style. This should lead to development of methods to overcome the incompatibility in *Protea* and, in doing so, promote the great potential of interspecific hybridization for breeding purposes. These methods might include application of growth-promoting substances to pistils, a shortening of the length of the style, pollination at different stages in the development of the style and stigma and crossing in both directions (Briggs and Knowles, 1967). The practicality and success of each of these methods should be investigated in pollination studies on different *Protea* species.

5.5 Literature cited

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Table 5.1 Pollen grain diameter of different *Protea* clones (mean ± se)

Clone	Species	Diameter (μm)
T 73 05 02	*P. neriifolia*	35.3 ± 1.16
'Cardinal'	*P. eximia* x *P. susannae*	34.5 ± 1.59
'Fiery Duchess'	*P. eximia*	33.2 ± 2.25
T 84 07 05	*P. magnifica*	32.7 ± 1.60
'Silk 'n Satin'	*P. neriifolia*	32.6 ± 1.64
'Red Robe'	*P. neriifolia*	31.8 ± 1.48
T 76 03 06	*P. eximia*	31.5 ± 1.38
'Florindina'	*P. cynaroides*	31.4 ± 1.20
'Aristocrat'	*P. aristata*	30.5 ± 1.57
T 88 11 05	*P. cynaroides*	30.4 ± 1.34
T 89 05 02	*P. repens*	30.1 ± 1.03
'Sneyd'	*P. repens*	29.5 ± 1.07
'Pink Ice'	*P. compacta* x *P. susannae*	29.5 ± 1.35
'Limelight'	*P. neriifolia*	28.8 ± 1.18
'Guerna'	*P. repens*	28.7 ± 1.24
'Rubens'	*P. repens*	28.1 ± 1.69
'Embers'	*P. repens*	28.0 ± 1.23
T 74 05 02	*P. obtusifolia*	27.9 ± 1.30
'Sylvia'	*P. eximia* x *P. susannae*	27.7 ± 1.15
'Sheila'	*P. magnifica* x *P. burchellii*	27.2 ± 1.72
T 94 06 05	*P. pudens* x *P. burchellii*	26.8 ± 1.29

Table 5.2 ***In vitro*** **pollen germinability (fertility) of interspecific *Protea* hybrids (mean ± se)**

Clone	Parents	% Germination	% Empty shells
T 75 09 04	*P. laurifolia* x *P.* ?	89 ± 3.05	1.9 ± 1.88
'Pinita'	*P. magnifica* x *P. longifolia*	85 ± 3.38	5.8 ± 1.68
T 83 01 07	*P. magnifica* x *P.* ?	84 ± 4.07	5.2 ± 1.17
'Niobe'	*P. laurifolia* x *P.* ?	84 ± 5.86	3.2 ± 1.25
'Liebencherry'	*P. repens* x *P. longifolia*	75 ± 4.75	2.3 ± 1.28
'Sheila'	*P. magnifica* x *P. burchellii*	73 ± 4.11	9 ± 8.97
'Patrysie'	*P. magnifica* x *P. obtusifolia*	68 ± 3.99	7.0 ± 1.77
'Lady Di'	*P. magnifica* x *P. compacta*	68 ± 4.30	0.3 ± 0.371
'Sylvia'	*P. eximia* x *P. susannae*	67 ± 7.38	21 ± 4.03
'Red Baron'	*P. compacta* x *P. obtusifolia*	64 ± 4.22	10.3 ± 2.52
'Satin Pink'	*P. longifolia* x *P. compacta*	62 ± 11.3	14 ± 14.1
'Pink Duke'	*P. eximia* x *P. compacta*	57 ± 5.12	0.19 ± 0.256
'Pink Ice'	*P. compacta* x *P. susannae*	51 ± 7.20	15 ± 6.08
'Princess'	*P. magnifica* x *P. laurifolia*	47 ± 5.19	25 ± 5.18
T 74 05 05	*P. compacta* x *P. burchellii*	47 ± 9.91	10 ± 4.61
'Susara'	*P. magnifica* x *P. susannae*	44 ± 4.70	10 ± 4.23
'Anneke'	*P. longifolia* x *P. neriifolia*	42 ± 8.37	36 ± 11.9
'Thomas'	*P. compacta x P. neriifolia*	39 ± 6.34	34 ± 9.37
T 84 06 04	*P. neriifolia x P. compacta*	36 ± 6.59	38 ± 7.04
'Cardinal'	*P. eximia x P. susannae*	33 ± 7.93	30 ± 4.07
'Brenda'	*P. compacta x P. burchellii*	33 ± 5.63	42 ± 3.39
'Pink Velvet'	*P. magnifica x P. compacta*	26 ± 7.73	17 ± 9.36
T 94 04 06	*P. glabra x P. laurifolia*	15 ± 5.22	47 ± 6.49
T 90 07 18	*P. cynaroides x P. grandiceps*	0 ± 0.00	100 ± 0.00
'Valentine'	*P. cynaroides x P. compacta*	0 ± 0.00	100 ± 0.00

Table 5.3 **Seed set in *P. eximia* cv. 'Fiery Duchess' after different pollination treatments**

Pollination treatment	% Seed set*
Cross-pollination with *P. eximia* hybrid 'Cardinal'	10.4[a]
Self-pollination	8.6[a]
Cross-pollination (intraspecific)	7.9[a]
Cross-pollination (interspecific)	1.3[b]
Control (autogamy)	0.0[b]
LSD 5%	4.23

*Values followed by the same superscript do not differ significantly (P = 0.05).

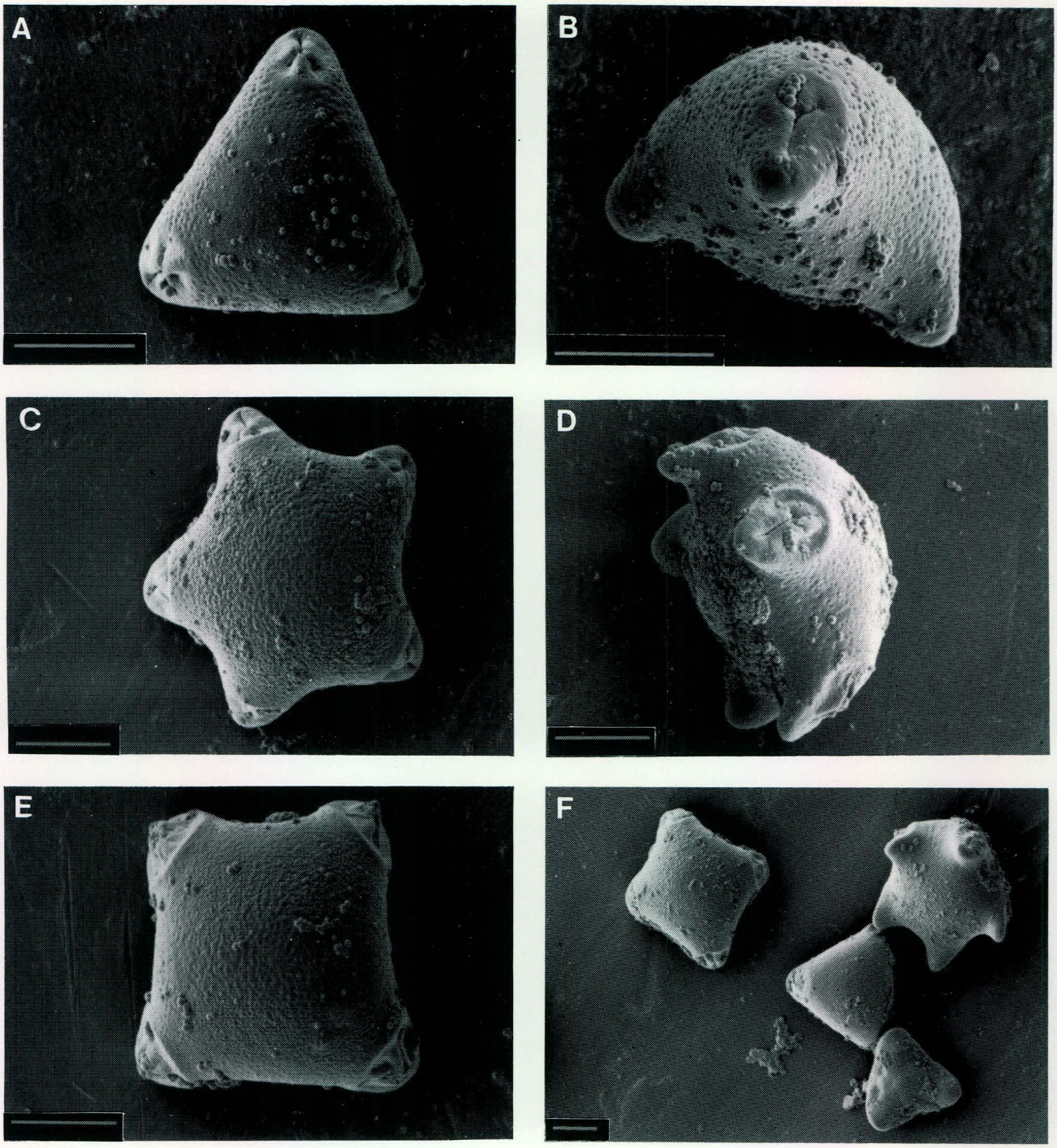

Fig. 5.1 Scanning electron micrographs of *Protea* pollen. A. Micrograph illustrating the general triporate triangular nature of the grains. x 2300. B. Micrograph of equatorial view, showing the aperture. Orbicules are clearly visible. x 3000. C. Micrograph showing the unusual quinquangular (5-porate) grain observed in pollen of cv. 'Red Baron' and cv. 'Patrysie'. x 1800. D. Micrograph of the quinquangular grain in equatorial view, showing the aperture. x 1800. E. Micrograph showing the unusual quadrangular (4-porate) grain observed in pollen of cv. 'Red Baron' and cv. 'Patrysie'. x 2000. F. Micrograph illustrating all three types of polymorphic grains observed in cv. 'Red Baron' and cv. 'Patrysie'. x 850. All bars = 10 μm.

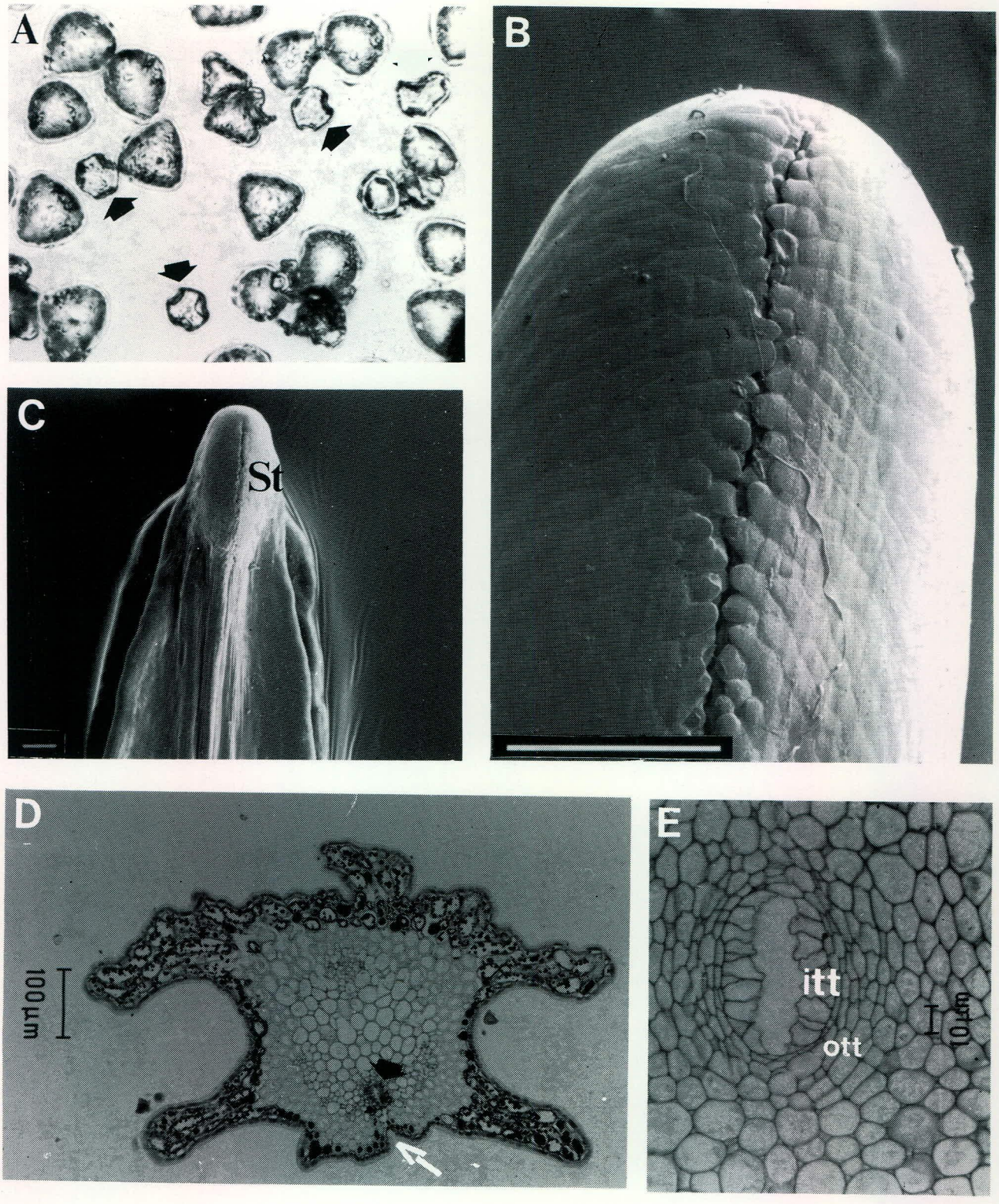

Fig. 5.2 A. Light micrograph of cv. 'Sylvia' pollen, showing a high percentage sterile grains (arrows). x 400. B. Scanning electron micrograph of the stigma of cv. 'Sneyd' , showing the serrated and interlocking nature of the stigma papillae cells. x 270, Bar = 100 μm. C. Scanning electron micrograph of the pollen presenter area of cv. 'Sneyd', showing the elongated ridged structure and the stigmatic groove (St). x 70, Bar = 100 μm. D. Transverse section (TS) of the pollen presenter area, stained with alcian green-safranin, showing the vertebra-shape, the stigmatic groove (thin arrow), and the stylar canal (thick arrow). x 100. E. TS of stylar canal, stained with alcian green-safranin, showing the densely-packed outer transmitting tissue (ott) cells and the two files of large inner transmitting tissue (itt) cells. x 400.

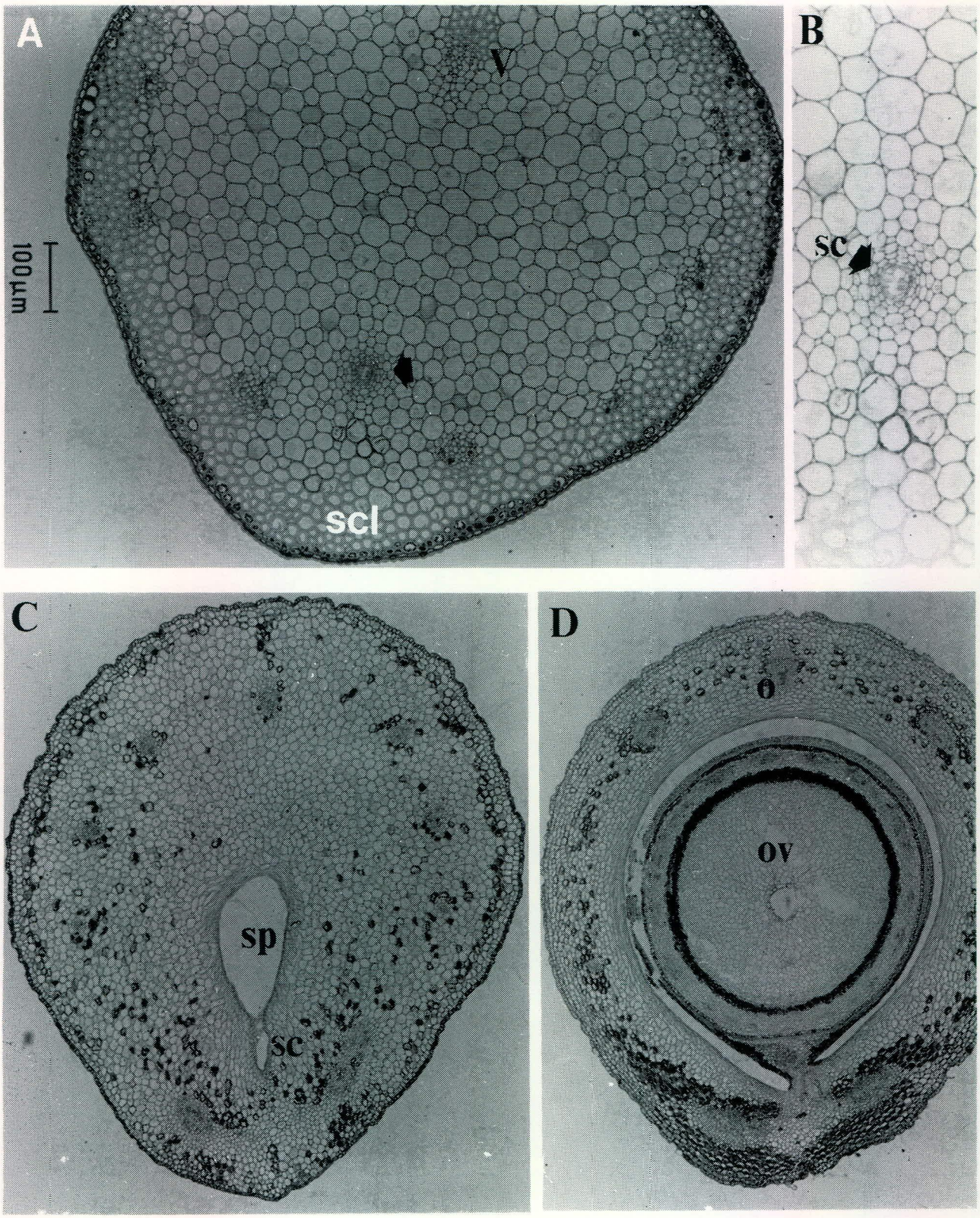

Fig. 5.3 A. Transverse section (TS) of the cv. 'Sneyd' pistil, 40 mm from the tip, stained with alcian green-safranin, showing the sclerenchyma cells (scl), the stylar canal (arrow), and the major vascular bundle (V). x 100. B. Detail of (A), showing the stylar canal (sc). x 200. C. TS of the beginning of the ovary, stained with alcian green-safranin, showing the stylar canal (sc) joining up with the cavity (sp) formed between the ovule and inner ovary wall. Polyphenol-containing cells (dark) are distributed throughout the cortex. x 40. D. TS through the ovary, stained with alcian green-safranin, showing the attachment of the ovule (ov) to the ovary (o). x 40.

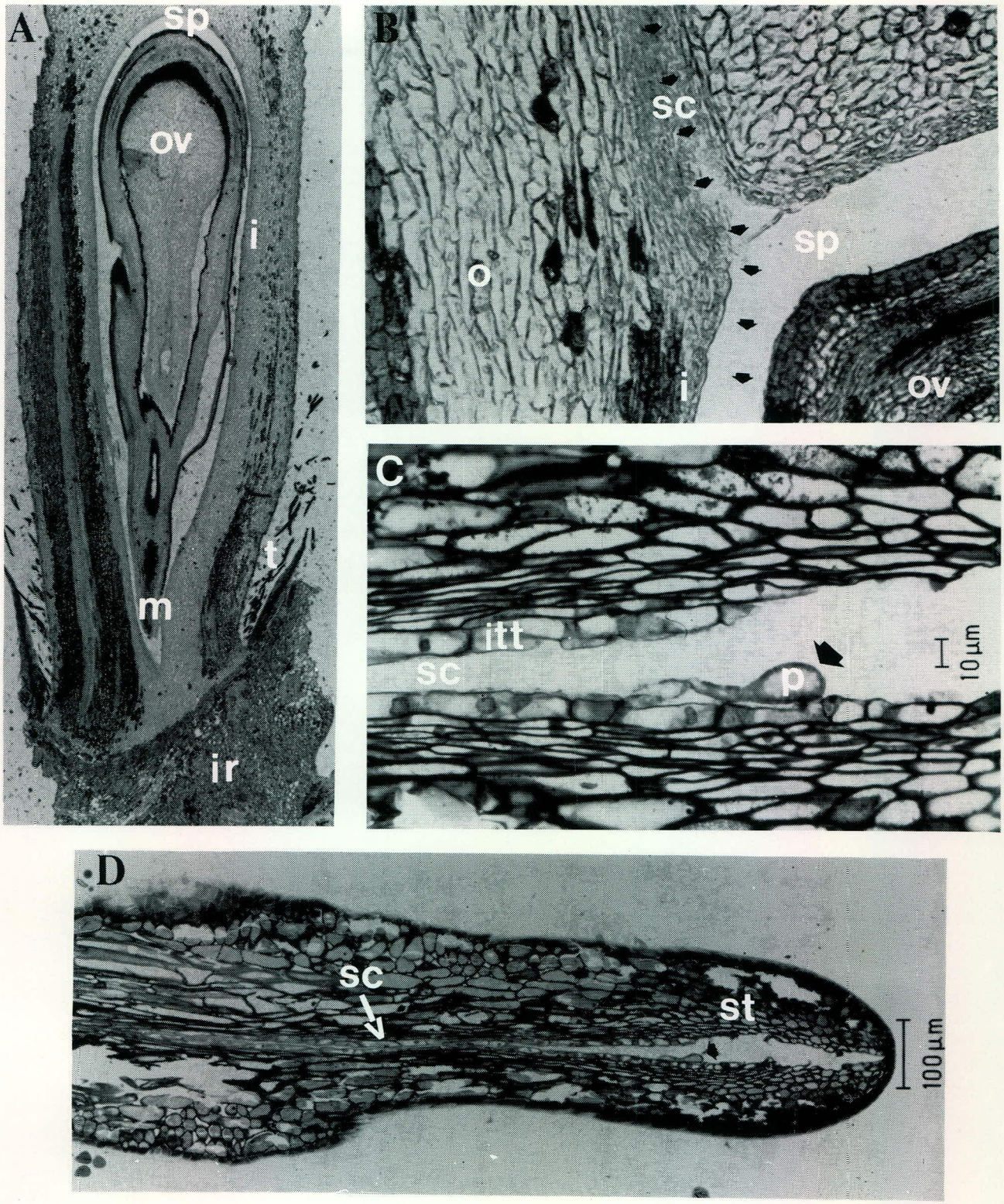

Fig. 5.4 A. longitudinal section (LS) through the ovary of cv. 'Sneyd', stained with alcian green-safranin, showing the single acutely obovate-shaped ovule (ov), cavity (sp) between ovule (ov) and inner ovary wall (i), micropyle (m), involucral receptacle (ir), and trichomes (t). x 20. B. Transverse section (TS) through the ovary (o), showing the stylar canal (sc) joining up with the cavity (sp) formed between the ovule (ov) and the inner ovary wall (i). The pollen tube pathway is indicated by arrows. C. LS through the pollen presenter area, stained with Toluidine Blue O, showing a germinated pollen grain (p), stylar canal (sc) and inner transmitting tissue cells (itt) lining the canal. x 400. D. The same as (C), also showing the stigmatic groove (st). x 100.

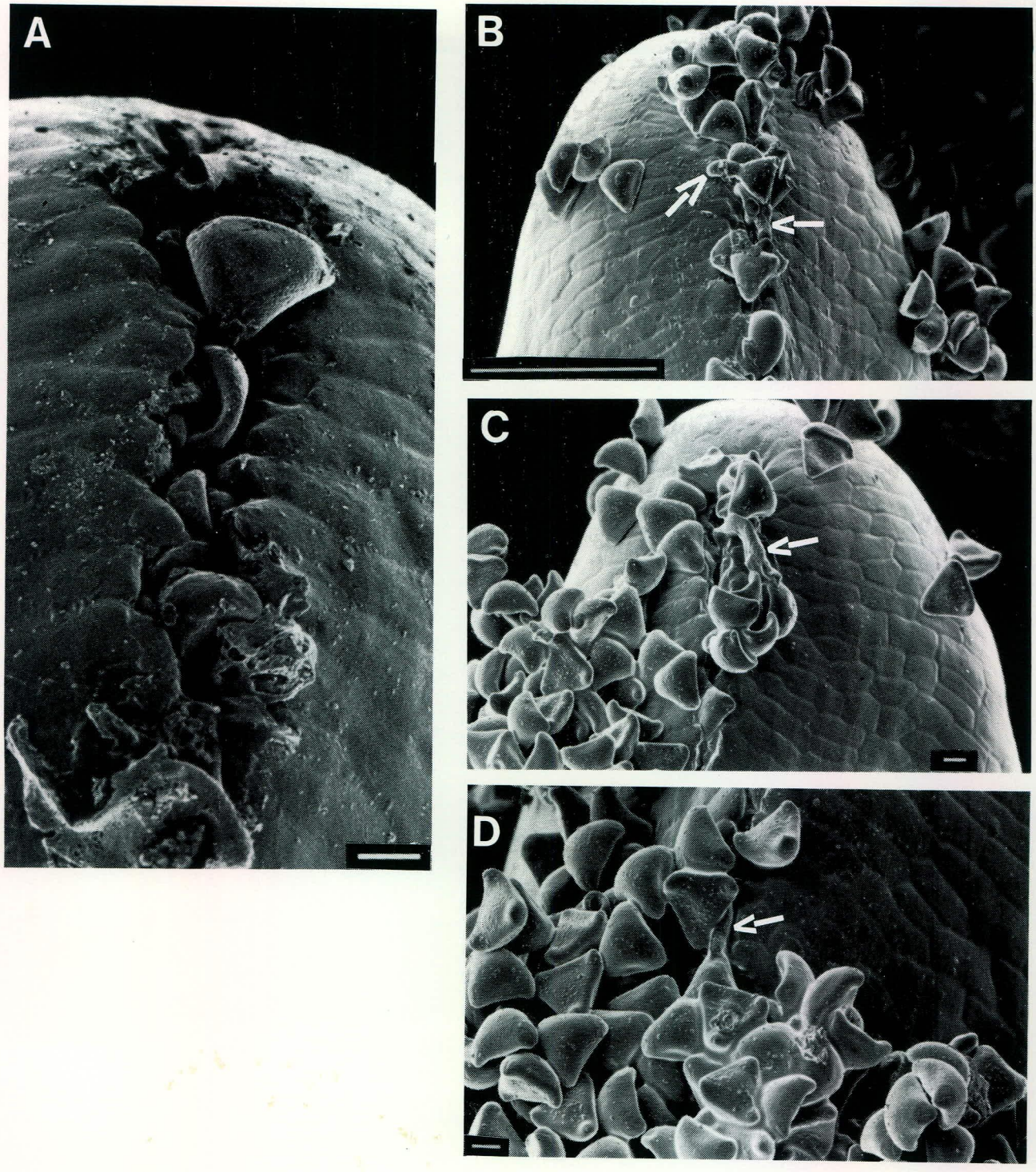

Fig. 5.5 A. Scanning electron micrograph of the stigmatic groove of cv. 'Sneyd', showing pollen grains wedged into the groove. x 800, Bar = 10 μm. B – D. Scanning electron micrographs of the stigmatic groove of cv. 'Sneyd' showing pollen grains germinating (arrows) and growing into the stigmatic groove. B, x 370, Bar = 100 μm. C, x 430, Bar = 10 μm. D, x 550, Bar = 10 μm.

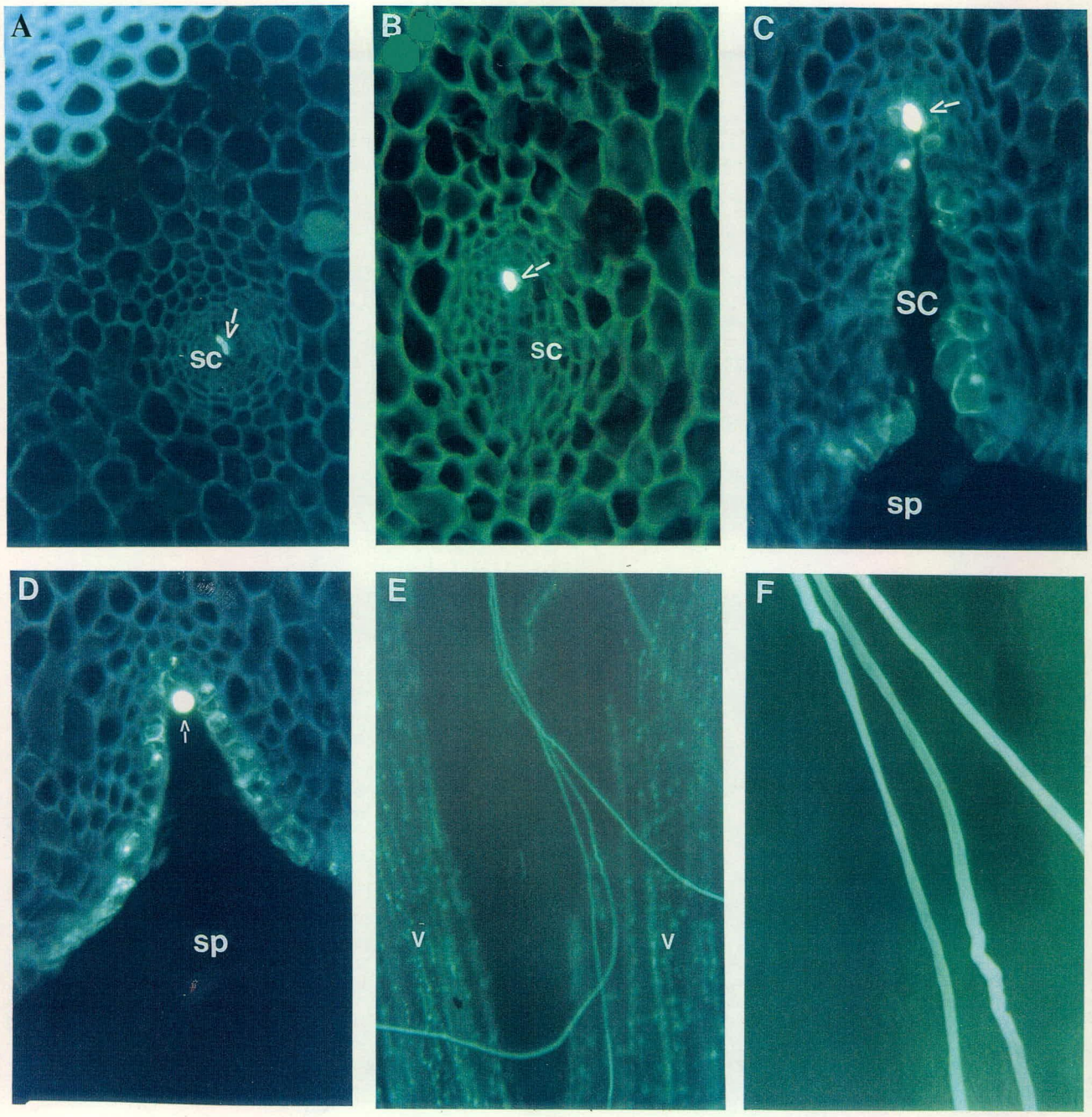

Fig.5.6 Fluorescence micrographs of pollen tube growth in *Protea*. A. Transverse section (TS) through the pistil, 40 mm from the tip, showing two pollen tubes (arrow) between the two files of inner transmitting tissue cells of the stylar canal (sc). x 160. B. TS through the pistil, 70 mm from the tip, showing one pollen tube (arrow) in the stylar canal (sc). x 160. C – D. TS through the pistil, showing one pollen tube (arrow) in the stylar canal (sc), at the point where the stylar canal joins up with the cavity (sp), formed between the ovule and the inner ovary wall. x 160. E. Micrograph of a squash preparation of the ovary, showing three pollen tubes growing down to the ovule. The strong autofluorescence of vascular bundles (V) are clearly visible in the back-ground. x 40. F. A higher magnification of (E), showing the uniformily distributed callose along the entire length of the pollen tube wall. x 160.

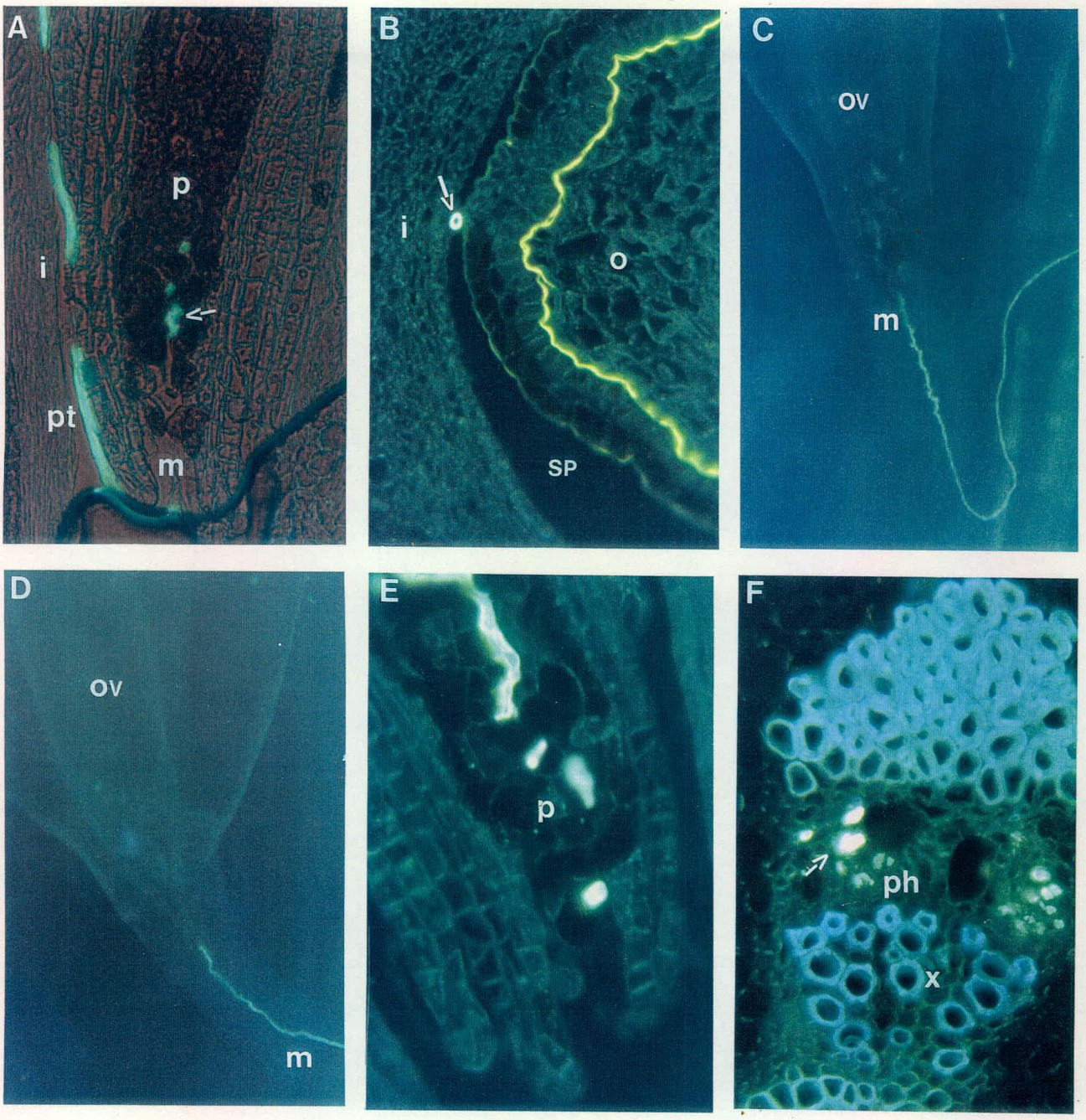

Fig. 5.7 A. Fluorescence micrographs of pollen tube growth in *Protea*. A. Longitudinal section (LS) through the ovary, showing a pollen tube (pt) growing in the cavity formed between the inner ovary wall (i) and the outer integument of the ovule, down to the micropyle (m). A piece of pollen tube (arrow) can be seen growing between the papillate cells (p) of the apex of the nucellus. x 160. B. Transverse section (TS) through the ovary, showing a pollen tube (arrow) growing in the cavity (sp) formed between the ovule (ov) and inner ovary wall (i). x 160. C – D. Micrographs of squash preparations, showing one pollen tube making a 180° turn and penetrating the micropyle (m). x 40. E. LS through bottom part of ovule, showing a pollen tube growing between the papillate cells (p) of the apex of the nucellus. x 400. F. TS through a vascular bundle showing the strong autofluorescence of the xylem (x), phloem (ph) and sieve plates (arrow). x 400.

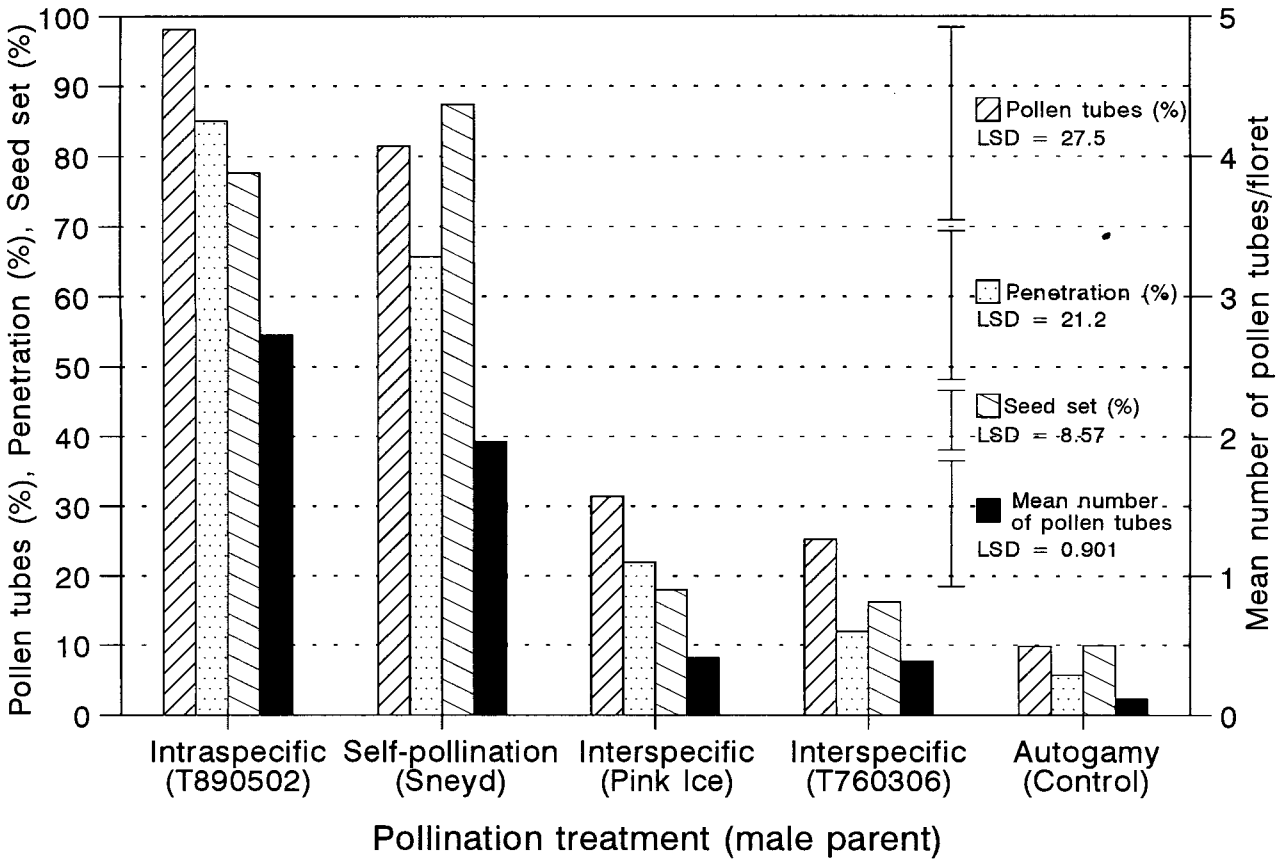

Fig. 5.8 Fertilization success of different pollination treatments as measured by: (1) percentage of florets with pollen tubes, (2) percentage of ovules penetrated by a pollen tube, (3) percentage seed set, and (4) mean number of pollen tubes per floret.

CHAPTER 6

STIGMA RECEPTIVITY OF TWO *PROTEA* CULTIVARS IN RELATION TO THE DEVELOPMENT OF HYBRIDIZATION TECHNIQUES

Abstract: Stigma receptivity of two *Protea* cultivars was investigated by means of observations on seed set, pollen tube growth and the opening and closing of the stigmatic groove. Both cultivars were found to be protandrous. Peak stigma receptivity as measured by seed set was found to be at two to six days after anthesis in cv. 'Sneyd' and at three days in cv. 'Fiery Duchess'. Seed set percentages were high in cv. 'Sneyd' and extremely low in cv. 'Fiery Duchess'. Peak stigma receptivity in cv. 'Sneyd' as measured by pollen tube growth was found to occur at two to six days after anthesis when the maximum mean number of pollen tubes per ovary was 2.5. Peak stigma receptivity in cv. 'Sneyd' based on the width of the stigmatic groove was found to be at three to six days after anthesis, and in cv. 'Fiery Duchess' at five to seven days. The maximum stigmatic groove width does not exceed the pollen grain diameter in these cultivars. Pollen tubes reach the ovary after four days and the maximum pollen tube number in the ovary occurs at seven days after anthesis. It is proposed that *Protea* spp. be hand-pollinated two to six days after anthesis in order to obtain maximum seed set. For studies on pollen tubes in the ovary, inflorescences should not be harvested before seven days after pollination.

6.1 Introduction

Ovule longevity, stigma receptivity and the rate of growth of pollen tubes in the style, are all factors which determine the period during which it is possible to successfully pollinate a flower (Egea *et al.*, 1991). However, no detailed investigations of the optimum time of stigma receptivity or *in vivo* growth rate of pollen have been made in *Protea*.

Protea, like most members of the Proteaceae, are protandrous (Vogts, 1971; Venkata Rao, 1971). Many Proteaceae, including *Protea*, exhibit a unique stylar presentation mechanism, where self-pollen is deposited on a specialized portion of the style, just before anthesis (Guthrie and Salter, 1950; Venkata Rao, 1971; Fuss and Sedgley, 1990). The stigma, containing the stigma papillae, is enclosed within a small stigmatic groove (Vogts, 1971; Brits and Van den Berg, 1990). In *Protea* the self-pollen does not cover the stigmatic groove. Pollination occurs when compatible pollen passes through the opening of the stigmatic groove to the stigma (Ramsey and Vaughton, 1991).

The most important aspects of stigma receptivity in the Proteaceae are the widening of the stigmatic groove and the secretion of stigmatic exudates (Sedgley *et al.*, 1985; Collins and Spice, 1986; Vaughton and Ramsey, 1991). The stigmatic grooves of most Proteaceae species investigated are almost closed at anthesis, and reach a maximum width 24 – 96 hours after anthesis (Brits and Van den Berg, 1990; Fuss and Sedgley, 1991a; b; Vaughton and Ramsey, 1991). Studies on stigma receptivity have included pollination experiments (Vaughton and Ramsey, 1991; Ramsey and Vaughton, 1991), morphological observations (Brits and Van den Berg, 1990; Fuss and Sedgley, 1991a; b), and esterase localization (Sedgley *et al.*, 1985; Collins and Spice, 1986).

Pollen tube growth can be measured by the transect method or by the length of the leading pollen tube at various times after pollination (Knox and Singh, 1987). Rates of pollen tube extension vary considerably among species (Heslop-Harrison *et al.*, 1984). Investigations of the pollen tube growth rate in *Macadamia* (Proteaceae) and *Banksia* spp. (Proteaceae) show that it takes anything from four to seven days for the longest pollen tubes to reach the ovary (Ito, 1980; Sedgley, 1983; Fuss and Sedgley, 1991a; b; Goldingay *et al.*, 1991).

The objective of this study was to find out when maximum stigma receptivity occurs, since it is essential to know when to pollinate florets.

6.2 Material and Methods

6.2.1 *Plant material*

All the stigma receptivity experiments were conducted during the 1993 and 1994 flowering seasons on two mature (seven-year-old) *Protea* cultivars, *P. repens* (L.) L. cv. 'Sneyd' and *P. eximia* (Salisb. ex Knight) Fourcade cv. 'Fiery Duchess', planted in experimental plantations at Elsenburg (latitude 33°51'S, longitude 18°50'E; 177 m a.s.l.) and Riviersonderend (latitude 34°08'S, longitude 19°54'E; 168 m a.s.l.) in South Africa. Prior to the experimental period all plants had been subject to routine plantation management practices, including drip-irrigation during the summer months. Harvesting of blooms in previous years served as the only form of pruning of the bushes. Cv. 'Sneyd' flowers during the late summer months from January to April while cv. 'Fiery Duchess' flowers during the spring months from September to November.

6.2.2 *Seed set*

Inflorescences of cv. 'Sneyd' and cv. 'Fiery Duchess' were prepared for pollination in the same way as described previously (Chapter 4). The inflorescences were recaged until pollination at 0, 1, 2, 3, 6 and 9 days after anthesis. Florets in the 0 day treatment were pollinated immediately. Controls were left unpollinated and caged. Florets were pollinated in the same way as described previously (Chapter 4). Ten inflorescences of cv. 'Sneyd' and nine inflorescences of cv.'Fiery Duchess' per treatment were pollinated with *P. repens* cv. 'Guerna' and *P. eximia* clone 'T85 02 03' respectively and recaged. Seven months after pollination when the achenes had matured (Van Staden, 1978), the seed heads were removed from the plants. The percentage seed set of pollinated florets per seed head was calculated in the same manner as described previously (Chapter 4).

6.2.3 *Pollen tube growth*

Twenty-one inflorescences (i.e., three replicates) of cv. 'Sneyd' were prepared for controlled hand pollination, pollinated, and recaged exactly as described above. Three inflorescences (c. 50 florets) per treatment were pollinated and controls were left unpollinated and caged. The inflorescences were left for seven days on the plants to allow compatible pollen tubes to penetrate to the base of the styles as preliminary observations had indicated that a six-day period is required for pollen tubes to reach the ovary part of the style. The ovaries of the pollinated pistils were carefully dissected out of the involucral receptacle and prepared for fluorescence microscopy as described in Chapter 4. The number of pollen tubes in the ovary, as well as the percentage of ovules which had been penetrated by pollen tubes were recorded.

6.2.4 *Width of stigmatic groove*

The width of the stigmatic groove of unpollinated pistils at 0 to 12 days after anthesis was examined with a Scanning electron microscope (SEM) in two separate experiments. In the experiment with cv. 'Sneyd', the stigmas were critical-point dried after fixation in 2.5% glutaraldehyde and dehydration in a graded ethanol series, mounted on aluminium stubs using double-sticking tape and silver paste, and sputter-coated with gold. Samples were examined with a Jeol JSM 6100 SEM operated at 5 kV. In the experiment with cv. 'Fiery Duchess', the stigmas were mounted on aluminium stubs using double-sticking tape and silver paste, frozen in nitrogen slush, and sputter-coated with gold in an Oxford CT 1500 Cryo-trans-system, before transfer to the cold stage of the Jeol JSM 6100 SEM operated at 4 kV. The width of the stigmatic groove at its widest point was measured from photographs or video prints of three to five samples per treatment (Fuss and Sedgley, 1991a; b).

6.2.5 *Experimental design and statistical analysis*

Two separate seed set experiments were carried out, each consisting of seven treatments in nine or ten randomized blocks. Separate analyses of variance were performed for each of the experiments after the data had been subjected to a working logit transformation. Back

transformations were made to present results in tabular form as mean percentage pollen germination. The pollen tube growth experiment consisted of three randomized blocks. Pearson product-moment correlations were calculated to measure the associations between the different variables. Two separate groove width experiments were carried out, consisting of six and nine treatments respectively in three to five randomized blocks. Separate analyses of variance were performed for these experiments. In all the experiments, analysis of variance was performed using SAS statistical software version 6.08 (SAS Institute Inc., Cary, NC, USA). Student's least significant differences (LSD) were calculated at the 5% level of probability to compare treatment means. For all other effects in the analysis of variance a probability level of 5% was considered significant.

6.3 Results

6.3.1 *Seed set*

Analysis of variance showed a significant treatment effect ($P < 0.01$) for both experiments. Results of the experiment with cv. 'Sneyd' are shown in Table 6.1 . Peak stigma receptivity based on the percentage seed set was found to occur during the period of two to six days after anthesis. Seed set declines rapidly thereafter. The one-day treatment yielded significantly fewer seed set compared to the two-day treatment, but did not differ significantly from the three- and six-day treatments. Pollination at anthesis (day 0) yielded only 33.8% seed set compared to 74.5% in the two-day treatment. The control (unpollinated) treatment yielded significantly fewer seed set (9.3%) compared to the pollination treatments.

Results of the experiment with cv. 'Fiery Duchess' are given in Table 6.2. Seed set percentages over all treatments were extremely low, but peak stigma receptivity based on the percentage seed set (8.7%) was at three days after anthesis. There were no significant differences among the other treatments (including the control).

6.3.2 *Pollen tube growth*

Results of the pollination experiment are given in Figure 6.1. Significant differences were found between the different pollination treatments for all three pollen tube variables tested. Very low numbers of pollen tubes reached the ovary part of the pistil, with rarely more than three pollen tubes per ovary. With exceptions of the mean number of pollen tubes in the sixth-day pollination treatment, the second-, third- and sixth-day treatments were not significantly different, and gave significantly better results than the other pollination treatments in respect of all three pollen tube variables tested. In all three cases, pollen tubes reached the ovary in 97 – 100% of ovaries observed and penetration of the ovule occurred in 89 – 97% of ovaries observed. The highest mean number of pollen tubes per ovary was 2.5 in the third-day pollination treatment. The control (unpollinated) had significantly lower pollen tube percentages in all treatments except for the mean number of pollen tubes of the nine and nil-day treatments. Correlation coefficients between the three pollen tube variables tested were highly significant ($P < 0.01$).

6.3.3 *Width of stigmatic groove*

Analysis of variance of stigmatic groove width showed significant treatment effects in both experiments. Maximum stigma receptivity of cv. 'Sneyd', based on the width of the stigmatic groove, was at three and six days after anthesis (Table 6.3) (Fig. 6.2). No stigma secretion was evident in cv. 'Sneyd' stigmas because of the critical-point drying treatment. The width of the stigmatic groove of cv. 'Fiery Duchess' increased to a maximum at five days after anthesis (Table 6.4) (Fig. 6.3). The groove of cv. 'Fiery Duchess' appeared to close at day six and opened wider the following day for reasons unknown. Build-up of stigma secretion from day three was evident (Fig. 6.3). The maximum groove width of cv. 'Fiery Duchess' (24.03 μm) was almost twice that of cv. 'Sneyd' (12.09 μm).

6.4 Discussion

The results of this study have shown that the cultivars of both species tested are protandrous as has been reported for *Protea cynaroides* (Vogts, 1971) and other proteaceous

species (Sedgley *et al.*, 1985; Collins and Spice, 1986; Brits and Van den Berg, 1990; Fuss and Sedgley, 1991a; b; Vaughton and Ramsey, 1991).

The percentage seed set of pollinated pistils of cv. 'Sneyd' was found to be relatively high over the full range of pollination treatments (> 27%) with a maximum of 74.5%. This is much higher than the 23.6% reported for open-pollinated *P. repens* in their natural habitat by Coetzee and Giliomee (1985). Supplementary irrigation of the experimental plants received or the controlled hand-pollinations in this experiment might explain this difference but not in the case of cv. 'Fiery Duchess' where seed set is < 10%. The apparently low seed set of this cultivar presents a challenging problem for future research.

Pollen tube data, like seed set, indicate that peak stigma receptivity occurrs two to six days after anthesis. The very low numbers of pollen tubes observed in this study are in agreement with other studies in the Proteaceae (Sedgley, 1983; Goldingay *et al.*, 1991; Fuss and Sedgley, 1991a; b). The percentage penetrated ovules appears to be proportional to the percentage of styles containing pollen tubes and to the mean number of pollen tubes in the ovary. This would indicate that high numbers of germinated pollen grains favour the growth of pollen tubes and their subsequent penetration of the ovary.

Peak stigma receptivity coincided with stigmatic groove opening in both cultivars, also in agreement with previous studies in Proteaceae (Vaughton and Ramsay, 1991; Fuss and Sedgley, 1991a; b) but the factors controlling the opening of the grooves in *Protea* are as yet not known. It appears unlikely that grooves open and close in response to touch (Ayre and Whelan, 1989), because the grooves open in the absence of any contact with the stigma. However, the opening of the grooves in *Protea* appears not to be as pronounced as in *Banksia* (Fuss and Sedgley, 1991a) and even at maximum width, the groove is narrower than the pollen-grain diameter.

A further important observation in these experiments was that many of the nine-day-old stigmas became discoloured (brown) and dry compared with those of younger florets. The poor seed set observed in those older pistils might be ascribable to senescence and loss of function.

This study has shown that the time of pollination after anthesis has a marked influence on the percentage seed set obtainable in *Protea* and that hand pollinations should be made on the day of peak stigma receptivity in order to maximize pollen germination and seed set. In the case of the two *Protea* cultivars investigated, the optimum period appears to be from two to six days after anthesis and should be investigated in other species of *Protea* as well.

6.5 Literature cited

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Table 6.1 **Stigma receptivity of *P. repens* cv. 'Sneyd' as measured by the percentage seed set after pollination**

Day of pollination (number of days after anthesis)	% Seed set*
0	33.8^{c}
1	55.9^{b}
2	74.5^{a}
3	63.7^{ab}
6	68.5^{ab}
9	27.2^{c}
control (unpollinated)	9.3^{d}
LSD (5%)	0.56

*Values (back transformed) followed by the same superscript do not differ significantly (P = 0.05; Student's LSD based on transformed data).

Table 6.2 **Stigma receptivity of *P. eximia* cv. 'Fiery Duchess' as measured by the percentage seed set after pollination**

Day of pollination (number of days after anthesis)	% Seed set[1]
0	3.6^{bc}
1	2.6^{bc}
2	3.6^{bc}
3	8.7^{a}
6	4.7^{b}
9	4.2^{b}
control (unpollinated)	2.1^{c}
LSD (5%)	0.66

[1]Values (back transformed) followed by the same superscript do not differ significantly (P = 0.05; Student's LSD based on transformed data).

Table 6.3 **Stigma receptivity of *P. repens* cv. 'Sneyd' as measured by the width of the stigmatic groove at different days after anthesis**

Days after anthesis	Stigmatic groove width (μm)$^{+}$
0	4.0^{c}
1	8.1^{b}
3	12.1^{a}
6	10.7^{a}
9	6.3^{b}
12	6.1^{bc}
LSD (5%)	2.28

$^{+}$Values followed by the same superscript do not differ significantly (P = 0.05).

Table 6.4 **Stigma receptivity of *P. eximia* cv. 'Fiery Duchess' as measured by the width of the stigmatic groove at different days after anthesis.**

Days after anthesis	Stigmatic groove width (μm)$^{+}$
0	4.8^{c}
1	7.9^{bc}
2	8.7^{bc}
3	11.1^{b}
4	11.6^{b}
5	24.0^{a}
6	12.6^{b}
7	19.2^{a}
9	12.6^{b}
LSD (5%)	5.87

$^{+}$Values followed by the same superscript do not differ significantly (P = 0.05).

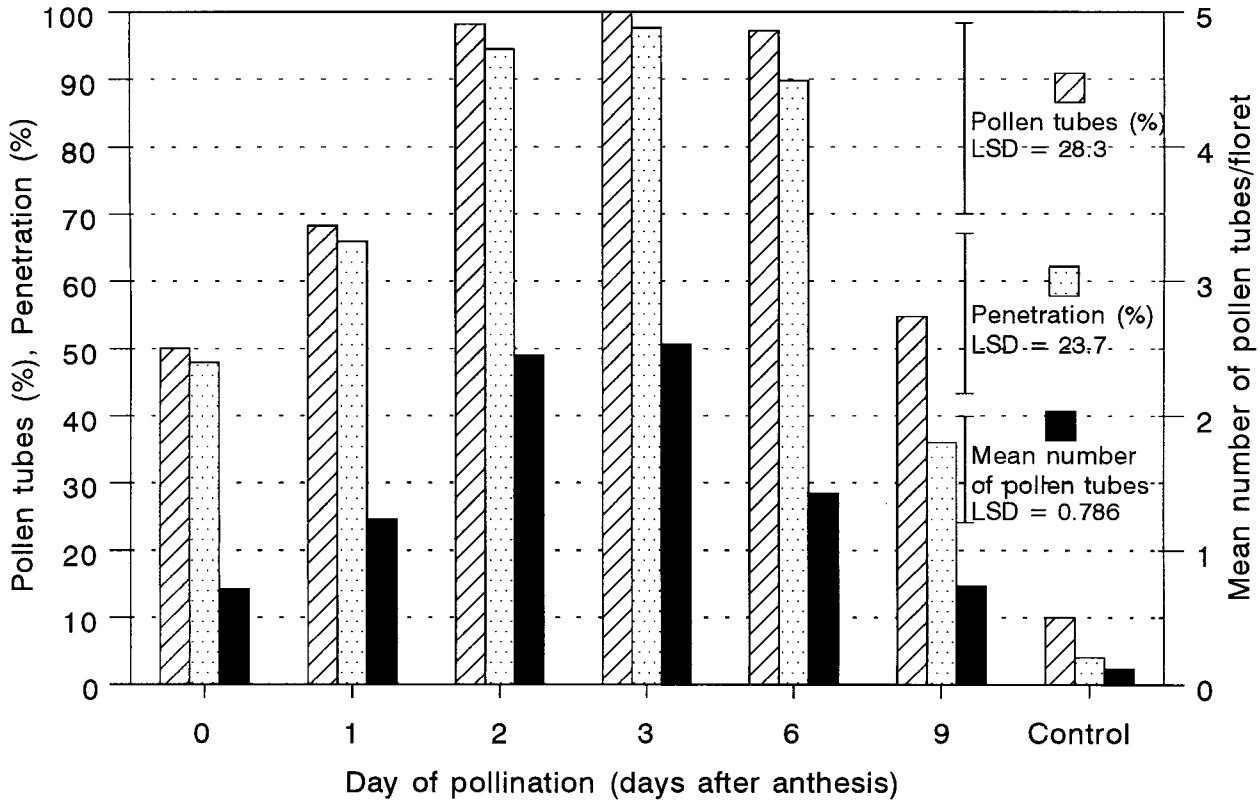

Fig. 6.1 Stigma receptivity of *P. repens* cv. 'Sneyd' as measured by: (1) percentage of florets with pollen tubes, (2) percentage of ovules penetrated by a pollen tube and (3) mean number of pollen tubes per floret.

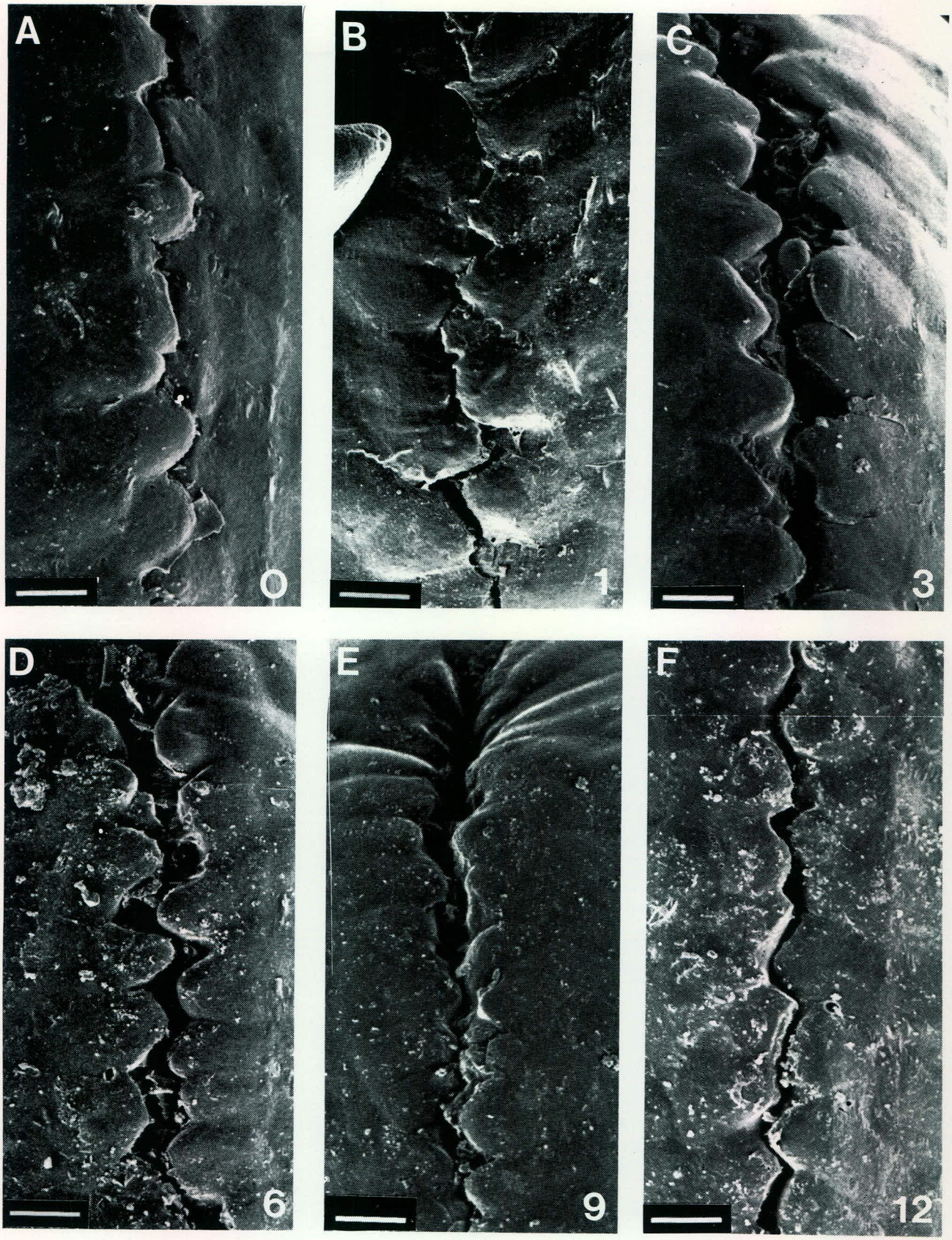

Fig. 6.2 Scanning electron micrographs of the stigmatic grooves of *P. repens* cv. 'Sneyd' at different days after anthesis. A. Groove at anthesis. B. Groove 1 d after anthesis. C. Groove 3 d after anthesis. D. Groove 6 d after anthesis. E. Groove 9 d after anthesis. F. Groove 12 d after anthesis. x 1000; Bars = 10 μm.

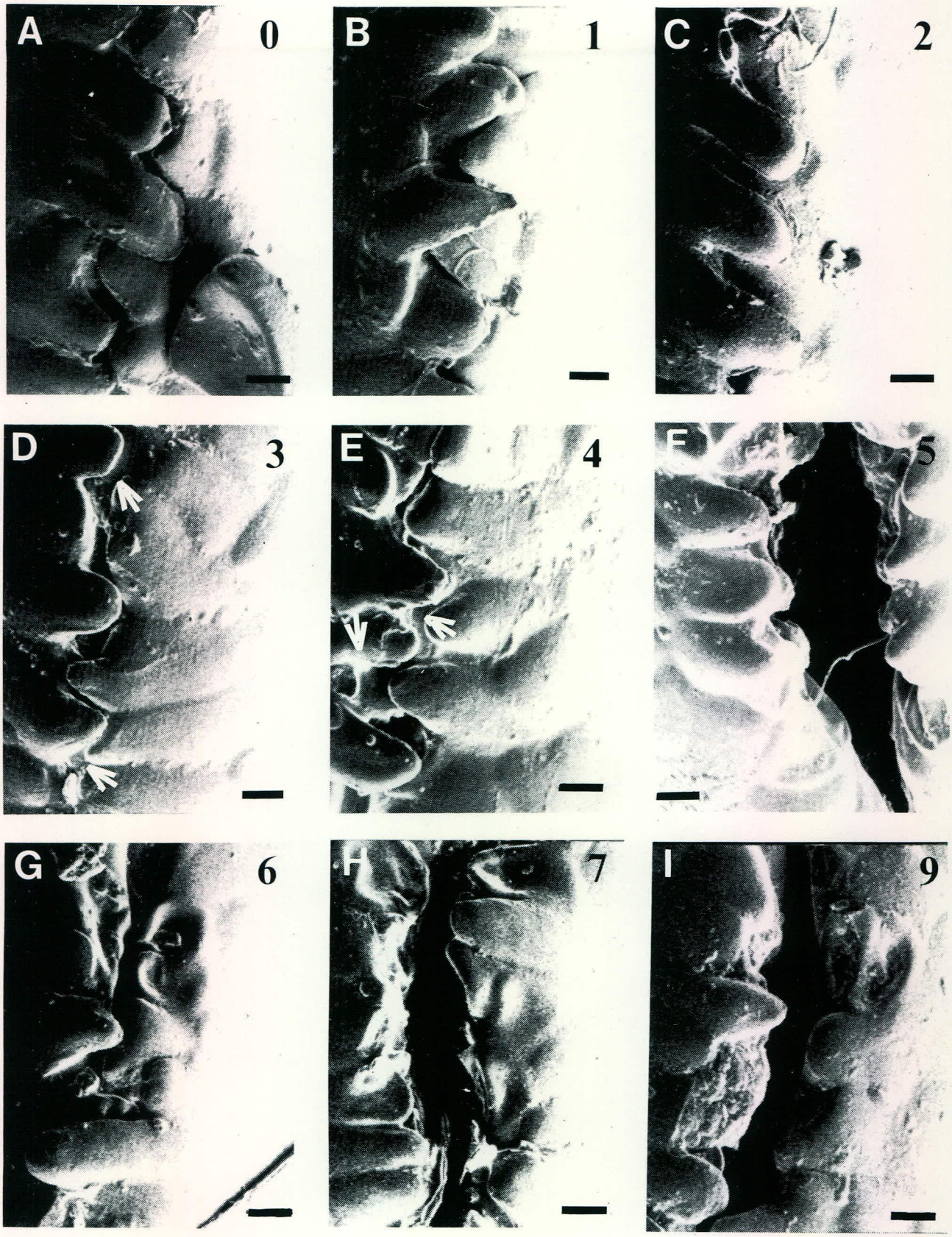

Fig. 6.3 Scanning electron video prints of the stigmatic grooves of *P. eximia* cv. 'Fiery Duchess' at different days after anthesis. A. Groove at anthesis. B. Groove 1 d after anthesis. C. Groove 2 d after anthesis. D. Groove 3 d after anthesis, showing small amounts of exudate (arrows). E. Groove 4 d after anthesis, showing larger amounts of exudate (arrows). F. Groove 5 d after anthesis, showing a large gap. G. Groove 6 d after anthesis, unexplainably closed. H. Groove 7 d after anthesis. I. Groove 9 d after anthesis. x 1000; Bars = 10 μm.